Molecular cloning of two molluscan caspases and gene functional analysis during *Crassostrea angulata* (Fujian oyster) larval metamorphosis

**Bingye Yang · Lingling Li · Fei Pu ·
Weiwei You · Heqing Huang · Caihuan Ke**

Abstract Caspases have been demonstrated to possess important functions in apoptosis and immune system in vertebrate. But there is less information reported on the oyster larval development. In the present work, two full-length molluscan caspase genes, named *Cacaspase-2* and *Cacaspase-3*, were characterized for the first time from Fujian oyster, *Crassostrea angulata*. Which respectively encode two predicted proteins both containing two caspase domains of p20 and p10 including the cysteine active site pentapeptide "QACRG" and the histidine active site signature. Otherwise *Cacaspase-2* also contains a caspase recruitment domain. Homology and phylogenetic analysis showed that *Cacaspase-2* shared high similarity with initiator *caspase-2* groups, but *Cacaspase-3* clustered together with executioner *caspase-3* groups. *Cacaspase-2* and *Cacaspase-3* mRNA were both highly expressed in gills and labial palp and were significantly expressed highly in larvae during settlement and metamorphosis. Through the whole mount in situ hybridization, the location of *Cacaspase-2* is in the foot of the oyster larvae and the location of *Cacaspase-3* is in both the foot and velum tissues. These results implied that *Cacaspase-2* and *Cacaspase-3* genes play a key role in the loss of foot and *Cacaspase-3* gene has an important function in the loss of velum during larvae metamorphosis in *C. angulata*.

Keywords Fujian oyster · *Crassostrea angulata* · *Cacaspase-2* · *Cacaspase-3* · Epinephrine · Metamorphosis · Apoptosis · Foot · Velum

B. Yang
Xiamen Medical College, Xiamen 361005, People's Republic of China
e-mail: yby128503@163.com

B. Yang · F. Pu · C. Ke (✉)
State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, People's Republic of China
e-mail: chke@xmu.edu.cn

B. Yang · F. Pu · W. You · C. Ke
College of Oceanography and Earth Science, Xiamen University, Xiamen 361005, Fujian Province, People's Republic of China

L. Li · H. Huang
College of Life Science, Xiamen University, Xiamen 361005, People's Republic of China

Introduction

The Fujian oyster, *Crassostrea angulata*, is an edible, cupped oyster of high commercial importance and wide distribution. During the oyster life cycle, the organism has a biphasic life cycle including pelagic larvae, and a benthic life that is morphologically distinct from planktonic larval forms [1]. *C. angulata* larvae typically metamorphose into juveniles simultaneously with or directly after settlement. Larval settlement and metamorphoses are among the most critical life periods, determining larval livability and the adult oyster environment. In the larval settlement and metamorphosis of oyster, dramatic change in morphology, physiology and habitat occur [2] and these changes are essential for the larva-to-adult transition, during which two of the most important morphological change are loss of the velum and the reorientation of the foot.

Apoptosis, programmed cell death characterized by a series of distinct morphological and biochemical alterations [3, 4], is a highly regulated and conserved active

cellular process essential for successful embryonic development and the maintenance of normal cellular and tissue homeostasis [5]. This is especially true for organisms which undergo metamorphosis-stage apoptosis. In amphibians, larvae both have marine and terrestrial habitats, during the amphibian life cycle cell apoptosis is critical for larval development like as the loss of tail [6, 7] and also cell apoptosis play an important role in regulation of insect larval metamorphosis [8, 9]. Moreover apoptosis has also been reported to occur during larval metamorphosis in marine invertebrates. During larval metamorphosis in *Ciona intestinalis* tail loss is regulated by apoptotic genes and apoptosis-related factors [10, 11], suggesting the necessity of apoptosis for larval metamorphosis. Even so, little has been reported about the relationship between apoptosis and oyster metamorphosis.

Caspases are critical for many apoptotic process stages [12, 13]. The cysteinyl aspartate proteases named caspases cleave substrates after aspartic acid residues which lead to protein degradation, finally running to cell death. Caspase proteins constitute the core of the apoptotic machinery [14], and as such have two main functions: death signal transduction and cellular protein cleavage, whereby they activate/inactivate many biochemical and morphological changes associated with apoptosis [15]. Depending on function, caspase proteins are categorized into two groups: apoptotic or inflammatory caspases. Then, apoptotic caspases can be further categorized as initiator apoptotic caspases and effector apoptotic caspases according to their functional site within the apoptotic cascade [16]. The initiator apoptotic caspases have long prodomains containing specific motifs such as death effector domains (DEDs) or caspase recruitment domains (CARDs) which are located up-stream in the caspase cascade and mediate apoptotic signals, cleaving and activating downstream effector caspases [17]. Effector caspases cleave various cellular substrates, leading to cell death [18, 19].

Here, we report that we cloned and characterized two novel caspase genes of initiator and executioner caspases from the Fujian oyster *C. angulatan*, a bivalve mollusk with high economical value and worldwide distribution. We also measured caspase gene expression in different tissues and larval developmental stages, and inspected caspase spatial location during early larval metamorphosis. We found that each type of caspase genes is selectively expressed in different tissues of the oyster larvae. These data will establish a foundation for studying the molecular mechanisms of mollusks larval metamorphosis and enable us to understand how the foot and velum retrogress during larval development.

Materials and methods

Samples collection and larva culture

Adult *C. angulata* were collected from the Xiamen coast and dissected to obtain different tissues including gills, visceral masses, female and male gonads, hemolymph, mantle, adductor muscles and labial palps. Samples were washed with $1 \times$ PBS, frozen in liquid nitrogen and stored at $-80\ ^{\circ}C$ until processed. Larval culture of C. *angulata* was conducted as previously described [20]. Larval samples were collected during the following stages: trochophore; D-veliger; umbo-veliger; larvae before settlement; larvae in metamorphosis (6 h after settlement); juveniles (2 days after settlement). Samples were washed with $1 \times$ PBS (0.01 M), frozen directly in liquid nitrogen and stored at $-80\ ^{\circ}C$ until processing. For whole-mount in situ hybridization (WMISH), larvae after 6 h of epinephrine treatment were collected and then fixed directly in 4 % paraformaldehyde overnight at 4 °C, but larvae were first anesthetized with gradual addition of $MgCl_2$ solution to the seawater in which the oysters were collected, and then tissues were collected for fixation. The trochophore and larvae were dehydrated across a methanol gradient and stored in 100 % methanol at $-20\ ^{\circ}C$.

RNA extraction and the first-strand synthesis

Total RNA of each sample was extracted with the RNAzol RNA isolation kit (Biotecx, Houston TX, USA) according to the manufacturer's instructions. The integrity and quantity of RNA was measured and total RNA was reverse-transcribed into cDNA as described previously [20]. First strand cDNA was synthesized and used as a template for further PCR analysis.

Molecular cloning of two caspase genes and sequence analysis

Cloning of full sequence caspase genes, 5′-RACE and 3′-RACE, were conducted separately using the Takara 5′-full RACE and 3′-full RACE cDNA Amplification Kit (Takara, Dalian, China) according to the manufacturer's instructions and the publication of Yang and coworkers [21] with the exception of the primers (primers for 5′-RACE and 3′-RACE are depicted in Table 1). In this study, the caspase-coding genes from C. angulata were designated as *caspase-2* and *caspase-3*. The two full caspase sequences were submitted to Genbank (accession no. JX890390 and JX890391).

To confirm sequencing accuracy of the caspase genes through RACE, two pairs of gene-specific primers (Table 1) were used for amplifying caspase cDNAs with

Table 1 List of primers sequences used in this study

Name	Experiment	Sequence (5′–3′)
caCaspase2YZ-F1	Confirm-PCR	TTTTGGTAACAGATGGAAGG
caCaspase2YZ-R1	Confirm-PCR	GTCAGACTGCGTAGGAAGC
caCaspase2-F1	RACE	GTGGTGTTCCCTTCCTTGA
caCaspase2-F2	RACE	GGTGTTCCCTTCCTTGATG
caCaspase2-R1	RACE	AAGGGAACACCACTGTCTAATG
caCaspase2-R1	RACE	TCCTTCCATCTGTTACCAAAA
caCaspase3YZ-F1	Confirm-PCR	ACATCGTGTCCTATTTCAA
caCaspase3YZ-R1	Confirm-PCR	ATCTATCAGCATATCCTCAA
caCaspase3-F1	RACE	CGTGGGTCGTCTCCTCAG
caCaspase3-F2	RACE	CTTATGATGAAGAGTGGCAGAC
caCaspase3-R1	RACE	CCTTCATTTGTCTGCCACTC
caCaspase3-R2	RACE	CTCTCTTTCACATCTATCAGCAT
18S-F	qRT-PCR	CGGGG AGGTA GTGACGAA
18S-R	qRT-PCR	ACCAG ACTTG CCCTC CAA
caCaspase2-F	qRT-PCR	ACCAGGCAGTTCTACGTCAC
caCaspase2-R	qRT-PCR	GAGTCAGCAGCAAACATTTC
caCaspase3-F	qRT-PCR	TTATGATGAAGAGTGGCAGAC
caCaspase3-R	qRT-PCR	AAACATTCGCGTGAGTTATG

polymerase Ex Taq (Takara, China) according to the following conditions: denaturation at 94 °C for 5 min, followed by 31 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 90 s. A final extension step was conducted at 72 °C for 10 min. The purified PCR products were cloned into a pMD-19T vector and transmitted into DH5α competent cells, plated on LB-agar flats, and eight independent clones were sequenced in both directions.

The entire nucleotide sequence was analyzed using BLAST from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). DNAMAN (DNAMAN Lynnon Biosoft, Santa Clara, USA) was used to identify its encoding protein. Prosite Server (http://expasy.org/prosite/) was used to predict the functional alleles of the gene. Amino acid sequences were aligned using the ClustalX (http://www.clustal.org/), and a phylogenetic tree was constructed using the Mega 4.1 program and the neighbor-joining method of clustering based on a PAM Matrix. The bootstrap value was computed over 1,000 replications.

Caspase expression analysis

Real-time qPCR was used to quantify changes in gene expression within different tissues and larvae samples during the development stages from trocophore to juvenile. The reverse transcription and real-time qPCR details are previously described [21]. The RT-qPCR reactions were conducted on ABI7500FAST. All samples were run in parallel with the housekeeping gene 18S rRNA. Primers for real-time qPCR are in Table 1. Data represent the means of three biological replicates, respectively. Data from competitive real-time PCR analysis subjected to one-way analysis of variance (ANOVA) followed by multiple comparison test with the LSD-t test was used to determine the differences in means with SPSS software. The P value for significance was set at $P \leq 0.05$.

Whole-mount in situ hybridization

Antisense and sense digoxigenin-labeled cRNA probes were synthesized with a DIG-RNA labeling Kit (Roche, USA). A PCR fragment related to *Ca-chit* was inserted to the PGEM-T EASY vector (Promega, USA), and then the ligation mixture was transformed into DH5α competent cells followed by sequencing. Plasmids were used as the template to amplify the DA$_{cga}$ cDNA fragment which was subjected to in vitro transcription. Riboprobes were synthesized by transcription with T7 and SP6 RNA polymerase and digoxigenin-11-UTP (Roche). Whole-mount in situ hybridization (WISH) was used for spatial expression analysis based on the protocol used in ascidiacea [22] with some modifications. WISH details have been described previously [20]. Images were taken with a digital camera (Olympus DP71) under a fluorescent light microscope (Olympus BX51). Digital photographs were imported into Adobe Photoshop CS, where they were cropped and the brightness and contrast were optimized.

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1      AAAATAATTCCCAAACAAAAAATCTGAACGACACGATGATTGTGGCAAGTCATCTAGTCTTTTTAACCGTAAAGTGGGCATACAACCGGT
91     TAACGGAAAAGTTCATGGGCCCTGCACCACCAAGCATGCAACATCAACTCCCTTTCCTCCTCCCGCACCAGCACCTGCACCCGGACTGAT
181    AATAAATCCTCATGACGCGGGTAGTTCGGTCGATCCCCCAAATTATCACGCTCAGCAACCGGCCTCTCGATCCTCGGTCCGACGACTACG
271    TTTAAGCAGCGGTGATCATATAAATGCGCGTCGGTTCTGATGCCGGGCTAGAGTTTGGAAAAGCAAATGACCTTCAACGCAATGACAATG
361    CTGTTTGGTCCTTGTGTCCTGAAAGGAAAACATGGCTCAATACATGAGTAGAGAAAGCAACAAGCCCGTCGAATACCACCTTATAAAAGA
451    GAACAAATAGAAGAGCACCAAAGCTTCAGGGATGAGTTAGTTACTGTTTGACCATCAGGGGTGGACTTTGATGATGGATAAGGCCAAGAA
1                                                                                       M  M D K A K K
541    GAACATCTTGATAAAGAACCGGGTGGAGCTGGTGGAGAATATCAACATAGAGGATGGACTGTTGACAGAGCTTCTGTCAAGACAGGTTCT
8        N  I  L  I  K  N  R  V  E  L  V  E  N  I  N  I  E  D  G  L  L  T  E  L  L  S  R  Q  V  L
631    CACCCAGAGGACCGCAAAAAGCATACAGGCAAAGAAGACTACATTTGACCAGACAGAGGAGCTTCTGGACAAAATAGCACTGAAAAGCAA
38       T  Q  R  T  A  K  S  I  Q  A  K  K  T  T  F  D  Q  T  E  E  L  L  D  K  I  A  L  K  S  N
721    TGGATTTGAAGCTCTGAGTGAGGCTTTGATAGCCAATGGCCAGGAAGACATTGTAAATCTCTATCTAAAACCACCTTCAGAAGAAAGTGA
68       G  F  E  A  L  S  E  A  L  I  A  N  G  Q  E  D  I  V  N  L  Y  L  K  P  P  S  E  E  S  E
811    AAAACTGAAGAGAGATGAAAAATCAGAGTCTGTAATATCGCAGGAATCTCAAACAGTTGGTCCAGAACCCACTCGTCCCCAGTCCTCTCC
98       K  L  K  R  D  E  K  S  E  S  V  I  S  Q  E  S  Q  T  V  G  P  E  P  T  R  P  Q  S  S  P
901    CCCCAGGCTCCCAGCCCCAGGGTCACAAGAGTCTCAACCAATCACCAAGGCAGTGCAACTCCTCACTCACACCGAAAGTGCCTCGCCCAG
128      P  R  L  P  A  P  G  S  Q  E  S  Q  P  I  T  K  A  V  Q  L  L  T  H  T  E  S  A  S  P  R
991    AGTCCTATCCCAGGGACAGCAGGAGTCTCAAGGAGTCCCCACCCGACAGCTCCAGCCTGTGTTACACCACCCACCACCAGATCATGTGTT
158      V  L  S  Q  G  Q  Q  E  S  Q  G  V  P  T  R  Q  L  Q  P  V  L  H  H  P  P  P  D  H  V  F
1081   CCACCCAATTCAGACCCACAGTGTTCAATCAGTGCCCGTCCAGCCTCCATCTGTTGATTCGTCCATTATTTGTGTGTCTGGAAATCCCGG
188      H  P  I  Q  T  H  S  V  Q  S  V  P  V  Q  P  P  S  V  D  S  S  I  I  C  V  S  G  N  P  G
1171   TCAGCCATACAACTTTAGCAATGCTGTGTTTTTGGATAGAGTCAGAAAGAGCGACGGGAAGCTTCAAGGAAAACCAACAACGACACAATTT
218      Q  P  Y  N  F  S  N  A  V  F  L  D  R  V  R  K  S  D  G  S  F  K  E  N  Q  Q  R  H  N  L
1261   AAAACACTATATCAACGACCCAAATTATCAGGTGCCCCCAGAGGTCCCTCCAACGACAGTGTCTCAACACATTCCTGTCATGTTCAATAA
248      K  H  Y  I  N  D  P  N  Y  Q  V  P  P  E  V  P  P  T  T  V  S  Q  H  I  P  V  M  F  N  N
1351   CAATGTTCATCCTGTTCCTAAAGAGGTTGTATACATTCCAAGTTACAGGCCAGCTTCTCTTCAAAGGGAGGATACTCTAGATGCTATGAC
278      N  V  H  P  V  P  K  E  V  V  Y  I  P  S  Y  R  P  A  S  L  Q  R  E  D  T  L  D  A  M  T
1441   CAAACCTTTGGGTAACCATGAGGCAAACTGTGAATCTCCAGCTAAAAGACAACGAACTGAGGAAAAAATCGAAATAACAGTGTTTGAATC
308      K  P  L  G  N  H  E  A  N  C  E  S  P  A  K  R  Q  R  T  E  E  K  I  E  I  T  V  F  E  S
1531   GAACCAGCCATCTCAGAATGAGCCCCAAATGATGACCCAGAGTAGCCTGCAGCCGGTGAGAATCCAGGGGGAGCTGGATGACCCTGAGAT
338      N  Q  P  S  Q  N  E  P  Q  M  M  T  Q  S  S  L  Q  P  V  R  I  Q  G  E  L  D  D  P  E  I
1621   AGATCTTACGGACGGGCCAGTTACCGTCAGAGTGGACAATACCACCAGGCAGTTCTACGTCACCAACTATAAGAAGTCCTATGCAATGAG
368      D  L  T  D  G  P  V  T  V  R  V  D  N  T  T  R  Q  F  Y  V  T  N  Y  K  K  S  Y  A  M  R
1711   GCGGTTGCCTCGAGGTAAGGCACTAATTATCAACGTGAATGAGGTGGAGGGGAAGCCACCCAGGCGAGGCACGGATATCGACAGGGATAA
398      R  L  P  R  G  K  A  L  I  I  N  V  N  E  V  E  G  K  P  P  R  R  G  T  D  I  D  R  D  N
1801   CCTGTATCACCTGCTGAGACAGCTCCACTTTGACGTCCAGGTGTATAACGACAAAGACGGACTCACTGCTAAGGAGATTGCTCAAAAATT
428      L  Y  H  L  L  R  Q  L  H  F  D  V  Q  V  Y  N  D  K  D  G  L  T  A  K  E  I  A  Q  K  L
1891   GGAAATGTTTGCTGCTGACTCCGCCCATCGCCAAGGAGATTCATCATTTGTGTGCCTTCTCAGCCATGGAGAGGAGGGTTTTATTTTTGG
458      E  M  F  A  A  D  S  A  H  R  Q  G  D  S  S  F  V  C  L  L  S  H  G  E  E  G  F  I  F  G
1981   AACAGATGGAAGGAAATTACAATTGGACAGTGTGTTCAAGCTGTTTGACAACAGCAATTGTCAACCCCTGGTGGGAAAACCAAAGATCTT
488      T  D  G  R  K  L  Q  L  D  S  V  F  K  L  F  D  N  S  N  C  Q  P  L  V  G  K  P  K  I  F
2071   CGTGATACAGGCTTGCAGAGGGGGAGCATTAGACAGTGGTGTTCCCTTCCTTGATGAACAAGATGGATCAACTGGATCTAAACAGCTTCC
518      V  I  Q  A  C  R  G  G  A  L  D  S  G  V  P  F  L  D  E  Q  D  G  S  T  G  S  K  Q  L  P
216    TACGCAGTCTGACATGATTATTTGCTTTCCTACACAAGCAGGTTACTATGCATGGAGGAATCGGGAGCGAGGCTGGTACATAGAGGC
548      T  Q  S  D  M  I  I  C  F  P  T  Q  A  G  Y  Y  A  W  R  N  R  E  R  G  S  W  Y  I  E  A
2251   GCTGGTACAAATCTTCATGAAGTACGCCAAGAGTGAGGACATTTGTACCATGCTGCACAGGGTAAACTTGCTGGTGTCAAGAAAAGTGTC
578      L  V  Q  I  F  M  K  Y  A  K  S  E  D  I  C  T  M  L  H  R  V  N  L  L  V  S  R  K  V  S
2341   CCGGTGTCCCCAAATAGAGATGGACTCCATGTCACAGATGTCGGAATACAAGAGCTCTCTACGCATGCCTCATCTTTTCTTCTATCCGGG
608      R  C  P  Q  I  E  M  D  S  M  S  Q  M  S  E  Y  K  S  S  L  R  M  P  H  L  F  F  Y  P  G
2431   GATCGGATGTGTTTAATCAAGCCCAAAGGGCACACTACAGTGGTGTGTACATGCATTGATGCAGAATATCTCTACAAATTTCTGAATGAAA
638      I  G  C  V  *
2521   AAAAAAAA
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◄**Fig. 1** The cDNA and deduced amino acid sequences (Genbank accession No. JX890390) of Caspase-2 from *C. angulata*. The CARD domains is underlined in *red* and the stop codon is indicated by "*asterisk*". The large P20 subunit and small P10 subunit are respectively underlined in *black* and *green*. The caspase family histidine active site and the pentapeptide in the cysteine active site are respectively indicated in *red* and *blue boxes*. (Color figure online)

Results

Sequence analysis of two caspase genes

In the present study, two molluscan caspase genes, *Cacaspase-2* and *Cacaspase-3*, were cloned for the first time from *C. angulata*. The full-length of *caspase-2* cDNA sequence was 2,528 bp, with a 1,923 bp open reading frame encoding a 641-amino acid protein (Fig. 1) that was ~72.1 kDa (estimated pI = 6.66). The full-length of *caspase-3* cDNA sequence was 1,381 bp, with a 1,215 bp open reading frame encoding a 404-amino acid protein

(Fig. 2) of ~46.1 kDa (estimated pI = 5.4). Another *caspase-3* gene named *Cgcaspase-3* from *C. gigas,* a sister subspecies of *C. angulata* also consists of an 1215 bp of open reading frame encoding a protein of 404 amino acids [23], the protein sequences of *Cacaspase-3* showed 96.78 % identity with the *Cgcaspase-3* and also have the similar domains including p20 and p10 domains. *Caspase-3* is an executioner caspases and ScanProsite analysis of the deduced amino acid sequence revealed that *caspase-2* and *caspase-3* both contained two domains of p20 and p10 which were conserved in the caspase family, including the cysteine-active site pentapeptide "QACRG" and the histidine-active site signature (Fig. 3). *Caspase-2* had one large N-terminal prodomain containing a CARD which is a 70-residue long structural motif and the caspase family histidine signature HTVYDCVVVIFLTHG was located between 466 and 480 amino acid residues (Fig. 1). These structures demonstrated typical caspase family characteristics.

Fig. 2 The cDNA and deduced amino acid sequences (Genbank accession No. JX890391) of Caspase-3 from *C. angulata*. The large P20 subunit and small P10 subunit are respectively underlined in *black* and *green* and the stop codon is indicated by "*asterisk*". The pentapeptide in the cysteine active site is indicated in *blue box*. (Color figure online)

C.Angulata-cas2	MMDKAKKNILIKNRVELVENINIEDGLLTELLSRQVLTQRTAKSIQAKKTTFDQTEELLDKIALKSNGFEALSEALIANGQEDIVNLYLKPPSEESEKLK	100
H. Sapiens-cas2	....	0
D. Rerio-cas2	....	0
C. Intestinalis-cas2	....	0
B. lanceolatum-cas2	....	0
H. Sapiens-cas3	....	0
B. lanceolatum-cas3	....	0
C.Angulata-cas3	....	0
D. Rerio-cas3	....	0
M.Galloprovincialis-cas3	....	0
Consensus		

C.Angulata-cas2	RDEKSESVISQESQTVGPEPTRPQSSPPRLPAPGSQESQPITKAVQLLTHTESASPRVLSQGQQESQGVPTRQLQFVLHHPPPDHVFHPIQTHSVQSVPV	200
H. Sapiens-cas2	....M	1
D. Rerio-cas2	....	0
C. Intestinalis-cas2	....	0
B. lanceolatum-cas2	....	0
H. Sapiens-cas3	....	0
B. lanceolatum-cas3	....	0
C.Angulata-cas3	....	0
D. Rerio-cas3	....	0
M.Galloprovincialis-cas3	....	0
Consensus		

C.Angulata-cas2	QPPSVDSSIICVSGNPGQPYNFSNAVFLDRVRKSDGSFKENQQRHNLKHYINDPNYCVPPEVPPTTVSQHIPVMFNNNVHPVPKEVVYIPSYRPASLQRE	300
H. Sapiens-cas2	AAPSAGSWSTFQHKELMAADRGRRILGVCGMHPHHQETLKKNRVVLAKQLLLSELLEHLLEKD.IITLEMRELIQAKVGSFSQNVELLNLLPKRGPQAFD	100
D. Rerio-cas2	............MLGECGMTKWERLALRRNSVKMLQDLVVDDLLIQCLQQDGILTDSMAESIMAKFTSQGRSHQLLFLLPKRGPRAFS	76
C. Intestinalis-cas2	............MKKRHRNTLIKNRVKLAKCLHLDEVLQYLVQCG.ILTDVTVERITSKPTRFSQNVAFLTCLPTRGPRAYG	69
B. lanceolatum-cas2	............MPQTKRPRQQFSDQSARGTDKTDWSIRFDTVVKEASHNWDGLAKELGFNQNEIKNIKSDVPDQARRCREILWKWKNKYGREA	82
H. Sapiens-cas3	....	0
B. lanceolatum-cas3	....	0
C.Angulata-cas3	............MATKKIPQEAKELHNELIADLHDNINSKVDEHDFEKLIHRLKLKLSPIDLKACHNLIDATDKLQKRGHLEPGNY	74
D. Rerio-cas3	....	0
M.Galloprovincialis-cas3	....	0
Consensus		

C.Angulata-cas2	DTLDAMTKPLGNHEANCESPAKRQRTEEKIEITVFESNQFSQNEPQMMTQSSLQPVRIQGELDDPEIDLTDGPVTVRVDNTTRQFYVTNYKK..SYAMRR	398
H. Sapiens-cas2	AFCEALRETKQGHLEDMLLTTLSG..LQHVLPPLSCDYDLSLPFFVCESCPLYKKLRLSTDTVEHSLDNKDGPVCLQVKPCTPEFYQTHFQL..AYRLQS	196
D. Rerio-cas2	TFCSALKETEQHHLCKLLMDFTE.........KDKCFSEPSLSLPTQECVTPAKRPR.THESMEMCLD.ADSPVTTAVLPCTPEFYQSHRPQ..AYPMRS	163
C. Intestinalis-cas2	TFLEALRISSQEHLVDILEKVDISNAIPNNKPITPTTLHATITSNVHGEFVCARRQFMEIGKCEDKNDAKLGHNRLRVQNSTVAFFNETYNS..GYKMTT	167
B. lanceolatum-cas2	TLEVLQQALVNIDEGDTAQALEEGAGPSNAVQEEGPRGGSTLDDPKPHHQDSAEPGAG..GNWEAPADEDDGKINVMVKTCDEEFWDKIMNEKDPYRMDS	180
H. Sapiens-cas3	............MENTENSVDS..........KSIKNLEPKIIHGSES....MDSG.......................MSWDTGY............KMDY	41
B. lanceolatum-cas3	.....MDDSCDAPAEDLFSPRLDS.......RDEHDAAPARGRAAAASADTTDAQKGASAGVMKFLG..IKGRSPAGISHGHRLGRRGLRL..CVNMNH	83
C.Angulata-cas3	GELIKLVDCIDKNWVNLIREKGET......IIEIIINKANEPQPPVSSEESERAKKRRLESDQDGQRSNENTDGQAQILKSSTKKERKKS.....VYPKTN	163
D. Rerio-cas3	............MN..GDCVDA.......KRVDTTDASKDGASASQPMQVDAKP.......................QSHAFRY.......SLNY	44
M.Galloprovincialis-cas3	.....MSKSDQRDSVPVTPEEQTS.....LKQGLMTNGIKQFERLLKKEYVCNSGP...............GTISKSHLN.......QDTYP	60
Consensus		

Large subunit

C.Angulata-cas2	LPRGKALIINVNEVEGK....PFRGTDIDRDNLYHLIRQ.LHFDVQVYNDKDGLTAKEIAQKLEMFAAD.SAHRQGDSSFVCLLSHGEEGFIFGTDGRK	492
H. Sapiens-cas2	RPRGLALVLSNVHFTGE.KELEFRSCGDVDHSTLVTLFKL.IGYDVHVLCD...QTAQEMQEKLQNFAQL.PAHRVTDSCIVALLSHGVEGAIYGVDGKL	290
D. Rerio-cas2	CPRGLALVLSNVRFDSANTDLDIRRGGEVDEETLRRLFTE.IDFKVSLHRD...LTAEAMRRCLEQFAQQ.QEHAAYDCAVVCLLSHGVEGSVYGTDGQL	258
C. Intestinalis-cas2	KSRGYALIVCIGRENAG.VRLPDRGTDADKKNLLAIFEQ.INYKTILVEN...CNKFEMERRVKQFAQM.EEHRKCDCSCAVAILSHGSKTDIYASDGRS	261
B. lanceolatum-cas2	KPRGYAILNNKNFTSL....PYRGGSAIDLSNITSLFQG.LSFETHILED..KKAQEIKGEVLAFSQR.KFHRQMDCCAVVLMSHGDEGVIFGTDDVP	271
H. Sapiens-cas3	PEMGLCIIINNFHKS.TGMTSRSGTDVDAANLRETFRN.LKYEVRNKND...LTREEIVELMRDVSK..EIHSKRSSFVCVLLSHGEEGIIFGTNG.P	133
B. lanceolatum-cas3	PRRGQAIIINNKDFEKH.TGMNTRRCTDLDANNLYRIFSK.IGFETKVFDN..LPCRKILSVLQLAAR..EIHRDVDCFACAILSHGEEGVIYGTDG.H	175
C.Angulata-cas3	DQRGLALILN...FTKG......RGANALFCGINDFFEKTLNFECHVETDPSKEEVECFLEETACLFNNKRTAAKYCLLVFIMSHGNEDGIRTNDGKT	254
D. Rerio-cas3	PNIGHCIIINNKNFDRR.TGMNFRNGTDVDAGNVMNVFRK.IGYIVKVYND...QTVAQIMQVLTTVAH..DIHSRCASLVCVLLSHGDEGVFFGIDT.S	136
M.Galloprovincialis-cas3	INAGIAVVINNIEFKRD.LGLPDRKGTDVDASSLFQRLQE.IGFDSDLLND...ASIEDMNEKFKEIKDDKEILKKTGCLIVALLTHGNEDSIYMTDV.S	154
Consensus	g	r g d	hg

C.Angulata-cas2	LQLDSVFKLEDNSNCQFLVGKPKIFIVQACRGGALDSGVPFLDE.....QDGSTG......SKQLFTQSDMIICFPTQACYTA	564
H. Sapiens-cas2	LQLQEVFQLFDNANCPSLQNKPKVFFIQACRGDETDRGVDQQDGKNHAGSPGCEESDAGKE......KLPKMRLFTRSDMICGYACLKCTAA	376
D. Rerio-cas2	LELDWVFEVSDNARCPLLQNKPKVFFIQACRGEEMDNVDQLDGQERTGSPGCEQRDAGREGERDNREKKEEKERERLRVKLFQRSDMICGFATLKGTAA	358
C. Intestinalis-cas2	IPIESLIKMSDNVNCPFLRNKPKIFIQCCRGNNMDQGIDATDGPPTLNSETSDSYNSTTHN....ITTDVDIISTASKRLFTSSDMIIGYATLQGNAA	356
B. lanceolatum-cas2	VQLDDIFAMSDNKNCPRLKGKPKVFFIQACRGLKVDKGVFVDEADGPGPNVKVNLKSEMRSFLHLP......EDESDGPEVQFTRTDMLCGYATQLGYHS	365
H. Sapiens-cas3	VDLKKITNFFRGDRCRSLTGKPKLFIIQACRGTELDCGIETDSG...VDDDMA.........CHKIPVDADFLYAYSTAPGYYS	205
B. lanceolatum-cas3	MEVTEVTKPFRGDRCPLVGKPKIFIQACRGNEFDHGVDMPDALPEVQDELDAGN..........KATIPAEADFLLAYSTVPGYYS	253
C.Angulata-cas3	ISLDDIVSYGKNDKLKYFAGKPKIFIVQACRGKISIFTDADLLLAYATTEGYFA	331
D. Rerio-cas3	VDLKSLTSLFRGDRCPSIVGKPKLFIIQACRGTELDPGVETDHP...DHPDIPDG..........RVRIFVEADFLYAYSTVPGYYS	210
M.Galloprovincialis-cas3	VKIRTIMDEFNATNCPELILKPKVFIIQACRGGGLGRGVNRKVIRDPNQADAGAEYDAYDEIYG............DTIRIFNEADFLAVVSTSAGYGS	241
Consensus	f	kpk f q crg	p d g

Small subunit

C.Angulata-cas2	WRNRERGSWYIEALVQIFMKYAKSEDICTMLHRVNLLVS.RKVSRCPQIEMDSMSQMSEYKSSTRMPHLFFYPGIGCV..	641
H. Sapiens-cas2	MRNTKRGSWYIEALAQVFSERACDMHVADMLVKVNALIK.DREGYAPGTEFHRCKEMSEYCSTLCR.HLYLFPGHPPT..	452
D. Rerio-cas2	MRNTKRGSWFIQELNTAIRQRANNTHLSDILVQVNGQIK.SREGYAPGSAHHRCKEMSEFTSSTCK.DLYLFPKYYPSN.	435
C. Intestinalis-cas2	LRNTRHGSWYIQVLVKKIAQHAHEMDLLEILTLVNSAIK.SKEGHSTDFDSQGVKEMSEFHSTLCK.KLFFYPGIVQTEK	434
B. lanceolatum-cas2	FRNTENGSWFIQAITKVFMEHAKDKSLTEMMPKVINDVS.KRTARCPGNPHHGGKEEAEFLYRLHK.PLYFFPGVE....	439
H. Sapiens-cas3	WRNSKDGSWFIQSLCAMLKQYADKLEFMHILTRVNRKVATEFESFSFDATFHAKKQIPCIVSMLTK.ELYFYH......	277
B. lanceolatum-cas3	WRNFPGRGSWFIQSLCAVLEREGTDLEIMQLITRVNKLVAYDFHSNSDNPYMNRKKQIPCIMSMLTK.DLFLSPKSQ...	328
C.Angulata-cas3	YRRPNVGSWFFCNLLEIFKEHYXEEHVEDMIIDVKERLAYDEEWQT....NEGFKQMPCTWSTLTK.RFFLAPQQLAE..	404
D. Rerio-cas3	WRNTMTGSWFIQSICEMMTKYGSELELLQIMTRVNHKVALDFESTSNMPGFDAKKQIPCIVSMLTK.EMYFTP......	282
M.Galloprovincialis-cas3	FRNTEKGSPFVRQLSEELLMKKDDDFYMILTRVNKRVGMGYKPNFPNSG..DVTQMPCFISHLTK.ELYLKQMN.....	313
Consensus	r gs	v	l

◄**Fig. 3** Multiple alignment of amino acid sequences of caCaspase-2,-3 and other caspase-2 s and -3 s. The caspase family histidine active site and the pentapeptide in the cysteine active site are respectively indicated by *blue* and *red boxes*. The amino acid *boxed in black* indicates conservation of identical residues in all sequences. The amino acid boxed in *pink* indicates conservation of residues with above 75 % consistency. Amino acid residues are numbered to the *right* of each sequence and *dots* represent indels. Caspase amino acid sequences are obtained from GenBank as follows: *Homo sapiens-cas2*, NP_116764.2; *Danio rerio-cas2*, NP_001036160.1; *Ciona intestinalis-cas2*, XP_002122917.1; *Branchiostoma floridae-cas2*, XP_002586743.1; *Homo sapiens-cas3*, CAC88866.1; *Branchiostoma floridae-cas3*, AAN45849.1; *Danio rerio-cas3*, CAX14649.1; *Mytilus galloprovincialis-cas3*, ADZ24781.1. (Color figure online)

Based on BLASTp results, *caspase-2* and *caspase-3* had maximal identity of 43.1 % with the marine worm *Capitella teleta* and 34 % identity with lancelet *Branchiostoma lanceolatum*. Phylogenetic analysis of the amino acid sequences between these and other caspases indicated that initiator caspases were divided into two main groups (Fig. 4): one branch included *caspase-2* and the second included *caspase-8, -9,* and *-10*. *Caspase-2* was placed into the *caspase-2* main branch, close to invertebrate sequences from groups such as lancelets and ascidiacea. Within the initiator caspases tree, all *caspase-8, -9,* and *-10* sequences as well as that of *caspase-2* from different species were almost clustered into different groups. Based on homology analyses of other caspases including *caspase-3, -7, -1, -4* and *-5* (Fig. 5), we found that all selected caspases were divided into two distinct clades in the phylogenetic tree, One clade (*caspase-3* and *-7*) belong to the executioner caspase superfamily. Another (*caspase-1,-4* and *-5*) group comprises the inflammatory caspases. *Cacaspase-3* was clustered together with an executioner caspase.

Tissues distribution of two caspases

Tissue-specific expression of caspase was analyzed using qRT-PCR in the various tissues of the normal oyster *C. crassostrea*. As shown in Fig. 6a, b, two caspase mRNA transcripts were expressed in all tissues but transcript abundance in each tissue varied. The most transcripts were detected in the labial palp and the gill followed by the mantle and visceral mass. Caspase gene transcripts revealed a relatively low expression in hemocytes, adductor muscles, and gonads.

Caspases expression in different developmental larval stages

Transcripts of mRNA from both caspases from the trochophore to the juvenile stage were also measured

(Fig. 7a, b) and caspase gene expression increased progressively over development, from D-shaped veliger larvae to metamorphosing larvae where it peaked. Expression of *caspase-2* in the trochophore was higher than in D-shaped veliger larvae.

Spatial–temporal expression of two caspases in larvae during metamorphosis

Through in situ hybridization (Fig. 8), *caspase-2* mRNA is located in the foot of the oyster larvae and *caspase-3* is found in the foot and velum tissues during larval metamorphosis.

Discussion

Many reports about caspases families suggest that they are critical for cell apoptosis or tissue, suggesting that caspases have central protease functions in apoptosis that cleave target proteins at specific sites with typical aspartic acid residues [24]. In *Mytilus galloprovincialis* and the gastropod *H. diversicolor*, some caspase genes were recently reported to be involved in immune defenses [25]. Few reports describe caspase genes in tissue loss during larval development in marine invertebrates. To address this deficit, we characterized two caspase genes in *C. angulata* and investigated their roles in the loss of the larval foot and velum.

Based on BLAST results and the prediction of structural domains, the two caspases sequence obtained had the caspase family signature and the conserved cysteine active site pentapeptide as well as the typical p20 and p10 domains of the caspase family. In addition, through phylogenetic analyses, the two caspases were classified as initiator and executioner caspases. One caspase had high identity to *caspase-2*, whereas the other caspase had high identity to *caspase-3*. *Caspase-2* and *-3* have high sequence similarity with those sequences found in other animal models [26, 27] and could be homologues to the *Capitella teleta* and *Branchiostoma lanceolatum* caspase genes, respectively.

The most important structural characteristic of initiator caspases was the presence of the CARD in *caspase-2*, which was similar to most other *caspase-2* proteins described in vertebrates [28]. And also the domains was similar to another *caspase-2* named as *Cgcaspase-2* from the oyster *Crassostrea gigas*, including CARD, P20 and P10 domains [29], but the protein sequences of two *caspase-2* was completely different, The *Cacaspase-2* showed only 12.14 % identity with the *Cgcaspase2*. The *Cacaspase-2* has the active-site pentapeptide QACRG (Fig. 1) and the typical p20 domain consistent with the full-length

functional *caspase-2* in humans [30]. This differs from the *caspase-2* in the mussel, which may be a splice variant of a full-length *caspase-2* [31]. Sokolova suggested that the death receptor-mediated apoptosis pathway may not be functional in nonchordate invertebrates [32], but the presence of the initiator domain CARD in oyster *caspase-2* and the CARD domain in *caspase-2* has been reported to

associate with death receptors [33], and previously reported descriptions exist regarding several proteins containing death domains from other invertebrates [34, 35]. These data suggest the possibility of a death receptor mediated pathway in invertebrates. One of our caspases belong to the *caspase-3* subtype which is a main executioner gene in apoptosis, responsible either partially or totally for

Fig. 4 Phylogenetic analysis of initiator caspases. Neighbor-joining (NJ) phylogenetic tree for initiator caspase proteins using MEGA 4.0. Caspase2 from *Crassostrea angulata* is indicated by *red ellipse*. *Numbers next to the branches* indicate bootstrap value of each internal branch in the phylogenetic tree nodes from 1,000 replicates. (Color figure online)
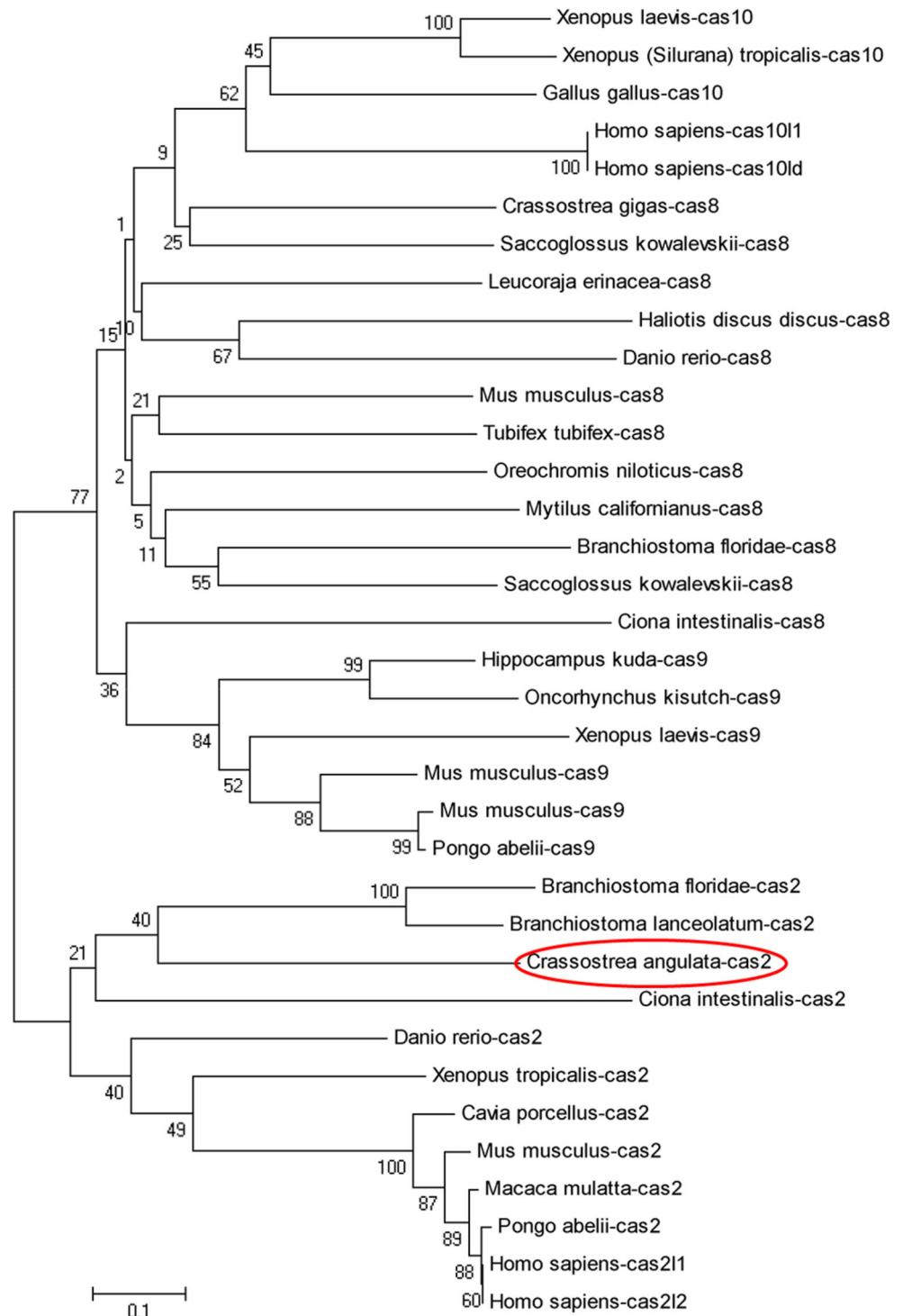
Fig. 5 Phylogenetic analysis of executioner caspases. Neighbor-joining (NJ) phylogenetic tree for executioner caspase proteins using MEGA 4.0. Caspase3 from *Crassostrea angulata* is indicated by *red ellipse*. *Numbers next to the branches* indicate bootstrap value of each internal branch in the phylogenetic tree nodes from 1,000 replicates. (Color figure online)
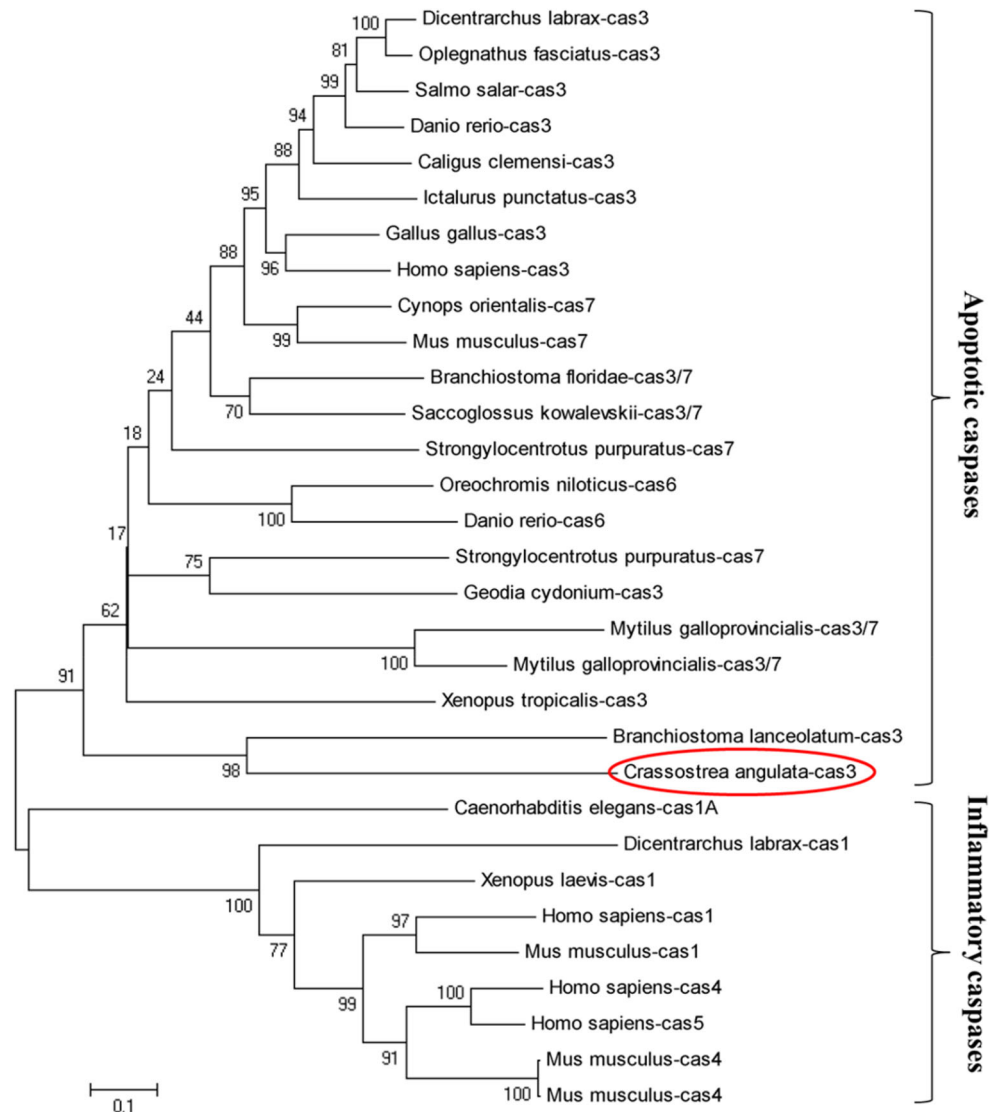
proteolytic cleavage of many key proteins, such as nuclear enzyme polymerases, which are cleaved in many different systems during apoptosis. *Caspase-3* contains short prodomains and both the conserved cysteine active site pentapeptide and the typical p20 and P10 domain structure are well described in mammals [36]. In addition, the critical amino acid residues of the executioner caspases involved in catalysis were well conserved among different species [37]. *Caspase-3* specifically degrades chromosomal DNA within the nuclei via activating of the endonuclease CAD and causes chromatin condensation [38]. *Caspase-3* also induces cytoskeletal reorganization and cell disintegration into apoptotic bodies.

Caspases are reported to be widely distributed in various tissues of invertebrates and vertebrates [19, 39, 40]. In our study, both *caspase-2* and *-3* mRNA were widely distributed in various tissues, indicating constitutive expression in

the adult oyster and a putative importance in eliminating undesired or injured cells, thereby fulfilling organismal development and homeostasis maintenance via regulating apoptosis [41]. Interestingly, caspase mRNA was expressed highly in the gill and labial palp which is consistent with *Cgcaspase-1* and *Cgcaspase-3* from *C.gigas* [23], indicating that caspases are probably involved in immune or metabolic processes in oyster, because gills and labial palp are the main tissues type involved in food and energy exchange, and the gills also is the chief tissues for respiration. Thus, injury to these tissues could be lethal; and, high caspase expression here might be protective. In addition, *caspase-3* expression was detected in hemocytes and lymphoid organs of white shrimp *Litopenaeus vannamei* [42], but in *C. angulata* we found that *caspase-3* expression was lower in hemocytes-a finding that is consistent with reports regarding the colored abalone [25]. In
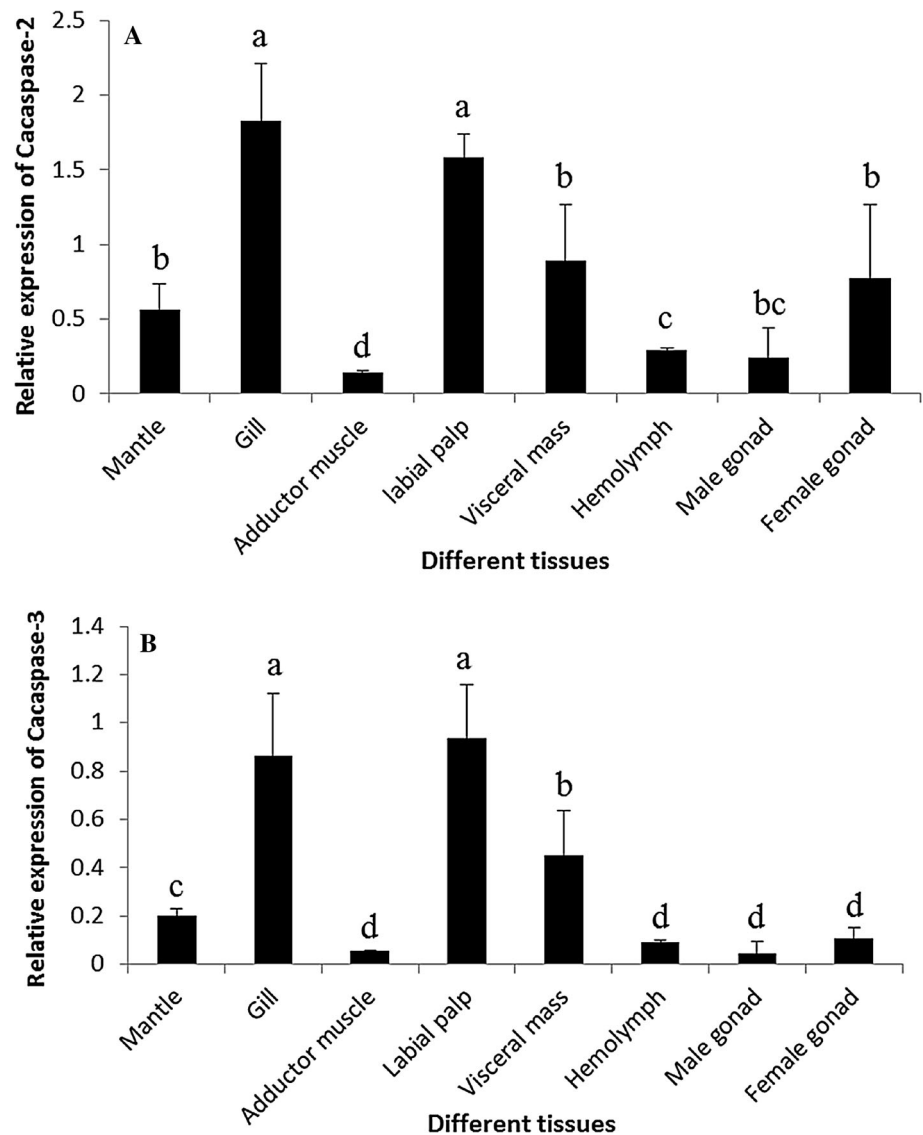
M. galloprovincialis, adductor muscle tissue had the lowest expression of most caspase genes [31], and these data were confirmed by our studies (Fig. 6). In addition, we also investigated caspase expression during larval developmental stages. The mRNA expression of *Cacaspase-2* in trochophore was higher than D-shaped larvae but the mRNA expression of *Cacaspase-3* did not increase, indicating that the initiator *Cacaspase-2* did not regulated the *Cacaspase-3* expression in the mRNA level through cascade reation in the Trocophore. The mRNA expression of *Cacaspase-3* were stable in Trocophore and the D-shaped larvae (Fig. 7), indicating that *Cacaspase-3* retained constitutive expression to maintain cellular homeostasis which is consistent with *Cgcaspase* [23]. We also noted that *Cacaspase-2* mRNA expression increased at an earlier time point than *Cacaspase-3*, meanwhile in larvae in settlement *Cacaspase-3* expression increased sharply comparing the

Cacaspase-2, finally the two *Cacaspase* achieved its peak in larvae of metamorphosis, which also conform to the expresssion description of the executioner caspase in *C.gigas*. These results implied that the initiator *Cacaspase-2* actavated at an earlier time point than the executioner *Cacaspase-3* in larvae of settlement and metamorphosis and both of *Cacaspases* play an important role in larval settlement and metamorphosis from *C. angulata*.

Caspase play a central role in apoptosis signals pathway. There are two major pathways for initiating apoptosis: an extrinsic pathway and intrinsic pathway, These two pathways activate the caspase cascade responsible for carrying out the orderly cell death programme. *Caspase-2* is an initiator caspase which can induce cytochrome-c release [43–45] and subsequent formation of an active complex of cytochrome-c with other apoptotic proteases [46], further activating *caspase-3* and causing cell death
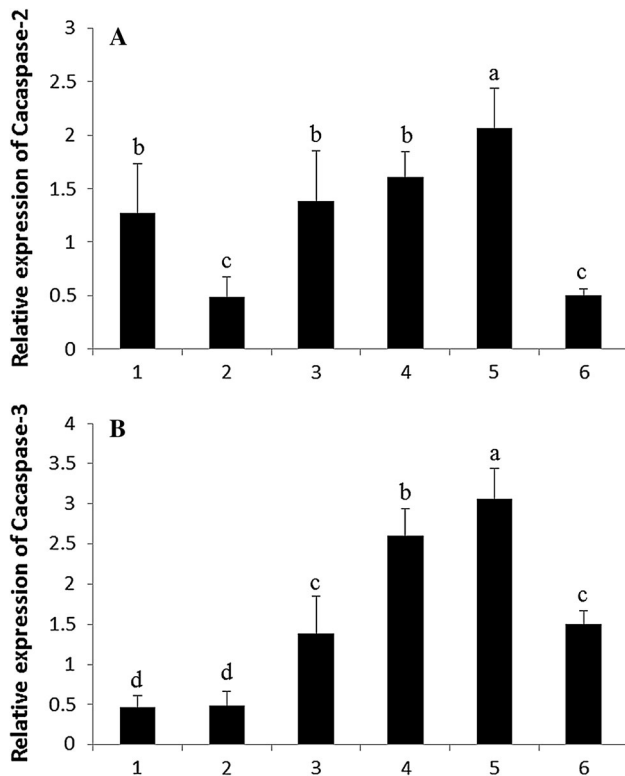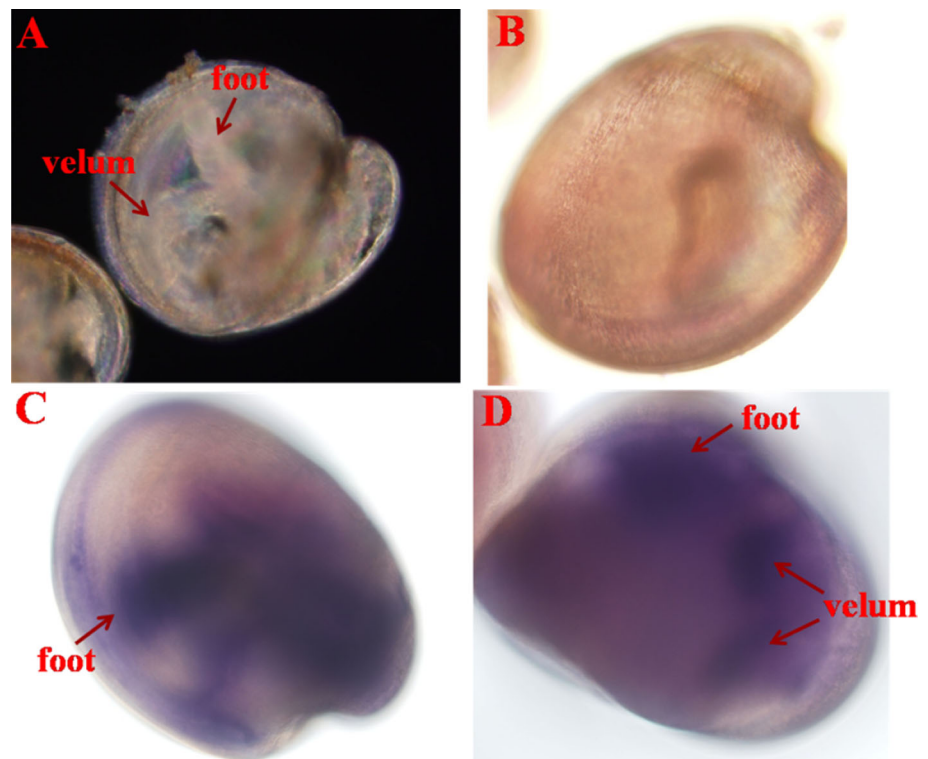
Fig. 7 Relative expression levels of caCaspase-2 (**a**) and caCaspase-3 (**b**) during larval development in *C. angulata*. Each *bar* represents the mean ± SD of three replicates. *1* Trocophore, *2* D-veliger, *3* umbo-veliger, *4* larvae in settlement, *5* larvae in metamorphosis (6 h after settlement), *6* juveniles (2 days after settlement)

[47]. As shown in Fig. 8, using whole-mount in situ hybridization, both *caspase-2* and *-3* mRNA was expressed in the foot during the early stage of larval metamorphosis in *C. angulata*, suggesting that a *caspase-2/caspase-3* pathway is involved in foot loss.

Early studies suggested that *caspase-2* was involved in nervous system apoptosis [48, 49], and the oyster foot contains the ganglion [50, 51]. Thus, this may explain high expression of *caspases-2* during metamorphosis. Our in situ hybridization data suggest that *caspases-3* mRNA located both in the foot and velum may be explained by apoptosis signals pathway coming together at the same point, causing cell death by activation of *caspase-3/7* [52]. *Caspase-2* mRNA was not highly expressed in the velum, suggesting that *caspase-2/caspase-3* was not involved in loss of this tissue. Therefore, caspase genes play an important role in the loss of the foot and velum and the *caspase-2/caspase-3* pathway is involved in foot loss.

In conclusion, two oyster caspase genes, *Cacaspase-2* and *Cacaspase-3* were characterized for the first time in *C. angulata*. One was an initiator caspases and the other was an executioner caspase. *Caspase-2* and *-3* mRNA were both expressed in various oyster tissues and were significantly expressed in larvae prior to metamorphosis. Through the whole in situ hybridization the results indicated that caCaspase genes play an important role in the loss of foot and velum and furthermore the *caspase-2/caspase-3* pathway was involved in the loss of foot.

Fig. 8 caCaspase-2 and caCaspase-3 mRNA expression profiles in the larvae in 6 h after epinephrine treatment by in situ hybridization with sense riboprobe (**b**) and antisense riboprobes (**c, d**), normal larva before metamorphosis (**a**)

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