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Original Research

Molecular cloning and characterization of genes encoding chitinase and N-acetyl-β-glucosaminidase in *Dolerocypris sinensis*

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Abstract: Chitinases and N-acetyl-β-glucosaminidase (NAG) are important in molting and growth of crustaceans. In ostracods, the genes encoding these enzymes have not been characterized. The aim of the present study was to clone the genes encoding chitinase (*DsChi)* and NAG (*DsNAG)* from the ostracod, *Dolerocypris sinensis,* elucidate the phylogenetic relationships between the cloned genes and known chitinolytic enzymes, and determine the expression patterns of these genes at different stages of growth in the presence of an environmental pollutant. The genes were amplified from the genomic DNA of the organism using polymerase chain reaction (PCR). The products from PCR were cloned and characterized with bioinformatics tools, and their expression patterns at different growth stages were determined using real-time quantitative PCR (qRT-PCR). Nine and five introns were identified in *DsChi* and *DsNAG* genes, respectively. When compared with protein sequences available in GenBank, chitinase from *D. sinensis* was most closely related to that of *Macrobrachium nipponense* (61 % homology). The NAG of *D. sinensis* was most closely related to that of *Limulus polyphemus* (55.6 % homology). Based on phylogenetic analysis of known chitinases from crustaceans and insects, the *D. sinensis* chitinase tightly clustered in the same branch with chitinases from species within the Malacostraca class. In contrast, NAG of *D. sinensis was* clustered with NAG from *F. candida*.The level of expression of *DsChi* mRNA was significantly higher than that of *DsNAG* throughout the period of growth ($p < 0.05$). Treatment of *D. sinensis* cells with fenoxycarb significantly downregulated the expressions of *DsChi* and *DsNAG* throughout the period of growth ($p < 0.05$). These results show that the protein products of *DsChi* and *DsNAG* possess remarkable biochemical properties characteristic of a chitinase and NAG, respectively.

Key words: *Dolerocypris sinensis*; Chitinase; Chitin; Acetyl-β-glucosaminidase; Molting.

Introduction

Chitin, the second most abundant component of biomass after cellulose, is a linear homopolymer made up of β-(1–4)-linked N-acetyl- β -glucosamine units, and serves as the principal structural component of the exoskeletons of insects and crustaceans (1, 2). Hydrolysis of chitin is catalyzed by a chitinolytic enzyme system comprising chitinase and NAG. Chitinase is an endoenzyme, while NAG is an exoenzyme (3). While chitinase cleaves glycosidic bonds in chitin, producing oligomers and trimers of β-(1–4)-linked N-acetyl-β-glucosamine, NAG hydrolyzes the terminal N-acetyl-D-hexosamine residues from these oligomers and trimers to yield the corresponding monomers (4, 5).

Both Chitinase and NAG are essential for the molting process during the growth of crustaceans. A chitinase-encoding gene was first identified in a crustacean species *Marsupenaeus japonicas,* and this prompted considerable efforts to identify similar genes in other crustaceans such as *Litopenaeus vannamei* (6, 7); *Fenneropenaeus chinensis* (8), *Portunus trituberculatus* (9), and *Eriocheir sinensis* (10). However, there are no reports on the identification of chitinase or NAG gene in ostracods which are important zooplanktonic members of the benthic community. These organisms are dominant in freshwater, and play crucial roles in the maintenance of the energy cycle in the ecosystem (11). Chitinolytic enzymes released into ambient water during molting of crustaceans are used as markers to estimate the biomass of crustaceans and the dynamics of their growth (12, 13). The expression of chitinolytic enzymes in crustaceans is inhibited by chemical waste products such as polycyclic aromatic hydrocarbons (PAHs) and fenoxycarb. The activities of chitinolytic enzymes are used for assessing ecological risks associated with environmental pollution (14). However, the dearth of genetic information on these ostracod enzymes has limited understanding of their biochemical properties and ability to properly harness them in tracking the impact of environmental pollutants. The aim of this study was to clone the genes encoding chitinase and NAG from the ostracod, *D. sinensis,* elucidate the phylogenetic relationships between the cloned genes and known chitinolytic enzymes, and determine the expression patterns of these genes at different stages of growth in the presence of an environmental pollutant.

Materials and Methods

Materials

NanoDrop ND2000 spectrophotometer was purchased from Thermo Fisher Scientific (USA); SV Total RNA Isolation System was obtained from Promega

(China), and Line Gene 9600 Plus system was a product of Bioer Technology. GoScript Reverse Transcription System Tool, pMD19-T Simple Vector, SMARTer RACE kit, and SYBR Premix Ex Taq II (TliRNaseH Plus) were obtained from Takara Clontech (China).

Collection of D. sinensis samples

Dolerocypris sinensis, was purchased from Shentankou Lake in Xixi Wetland, Hangzhou, China, and was identified by Professor Wang Xin-hua, Life Science College, Nankai University, Tianjin, China. The organism was acclimatized to the laboratory environment for more than three years. *Dolerocypris sinensis* was cultured in M4 medium supplemented with *Scenedesmus subspicatus* algae (1×10^6 cells/mL) (15). The laboratory conditions were: water temperature of 26 ± 2 °C, pH of 6.5 ± 0.3, and 17 h light/9 h dark cycles.

Extraction of DNA and RNA, and cDNA synthesis

Total RNA was extracted from *D. sinensis* using SV Total RNA Isolation System, while genomic DNA was extracted using hexadecyltrimethyl ammonium bromide (CTAB) method (16). The concentrations and quality of extracted RNA and DNA were assessed spectrophotometrically at 260/280 nm, and their integrity were determined electrophoretically on 1.3 % agarose gel. An aliquot of RNA extract corresponding to 6 µg was converted to cDNA using GoScript Reverse Transcription System Tool. Reverse transcription was carried out at 37 °C for 15 min and terminated at 85 °C for 5 sec.

Cloning of cDNA sequences

The cDNA sequences for the central region of each gene was cloned. The primer pairs used for amplifying the *DsChi* and *DsNAG* genes were Chi-F1–Chi-R1 and NAG-F1–NAG-R1, respectively (Table 1). The PCR cycling conditions were: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 63 °C for 30 sec, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were gel-purified and cloned into the pMD19-T Simple Vector. The resultant recombinant clones were screened using PCR and Sanger sequencing. After cloning the central region of the cDNA sequence of each gene, both ends were extended using rapid amplification of cDNA ends (RACE). The 5′- and 3’-RACE-ready cDNA templates were prepared using SMARTer RACE kit. The 5’-end cDNA was amplified with gene-specific primers (Chi-5’GSP1 and Chi-5’GSP2 for *DsChi,* and NAG-5’GSP3 for *DsNAG*) as reverse primers, while universal primer mix (UPM) from the SMARTer RACE kit was used as forward primer. The conditions for the first round of PCR were: pre-denaturation at 94 °C for 5 min, five cycles at 94 °C for 30 sec and 72 °C for 1.5 min, five cycles at 94 °C for 30 sec and 70 °C for 1.5 min, and 25 cycles at 94 °C for 30 sec and 66 °C for 1.5 min. The conditions for the second round of PCR were: pre-denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, and at 66 °C for 1.5 min. The 5’-end cDNA for chitinase was amplified with two rounds of PCR, while only the first round of PCR was performed for *DsNAG*. The 3’-end cDNA was amplified using gene-specific primers (Chi-3’GSP1 and Chi-3’GSP2 for *DsChi* and NAG-3’GSP3 and NAG-3’GSP4 for *DsNAG*) as forward primers. As usual, UPM was used as reverse primer. The conditions for the first round of PCR were: pre-denaturation at 95 °C for 2 min, five cycles at 95 °C for 30 sec, and at 72 °C for 30 sec, five cycles at 95 °C for 30 sec and at 70 °C for 30 min, 25 cycles at 94 °C for 30 sec and at 66 °C for 30 sec. The conditions for the second round of PCR were: pre-denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, and at 66 °C for 30

Table 1. Sequences of primers used in the PCR and RACE.

Experiments	Primers	Sequences (5′ to 3′)
Initial PCR cloning of cDNA fragments	Chi-F1	CAGGGGAAGAACCCACTCAT
	Chi-R1	GTCGGCGATTTTGGACGGTTT
	NAG-F1	AGAGGATTGCTCCTGGACAC
	NAG-R1	GCGTTCAGCGATGGCGCTAG
Rapid amplification of cDNA ends (RACE)	Chi-5′GSP1	GGGGCATTCGGATCCCTCGTGGTG
	Chi-5′GSP2	CCTTGAGCTTCTCATACATGAC
	Chi-3′GSP1	GCCCTGGACCAACTTGCACGCAGTA
	Chi-3′GSP2	CCGGAACTGGAGACGTGCTCCGAC
	NAG-5′GSP3	GTCCAGAGCGAGGAATATGTCTTC
	NAG-3′GSP3	CACGAACATCCATCCGCTGCTCTG
	NAG-3′GSP4	CTGTGGAGTTCAGCGGATGTTCG
Cloning of genomic sequences	Chi-F2	CCTAAAGCTCATCTATTGGTTTC
	Chi-R2	CTTGTGAGTCACACTCTTC
	NAG-F2	TCACAGCTTTCTTGTAATTG
	NAG-R2	TCGATGTATTCCATCCATAG
Reverse-transcription quantitative PCR	Actin-F	GGACAGAACGGCTTGGATGG
	Actin-R	ACCACACCTTCTACAACGAACTC
	Chi-F	GCCTCGTTCCTTACACTCACA
	Chi-R	CCTTCTCCTTAATCCAGTCCATCT
	NAG-F	AACATCCATCCGCTGCTCTG
	NAG-R	ACCACACCTTCTACAACGAACTC

Table 2. GenBank accession numbers for sequences used to construct phylogenetic trees.

Species	Taxonomy (Phylum, subphylum, class)	GenBank accession numbers	
		Chitinase	NAGase
*Dolerocypris sinensis**	Arthropoda, Crustacea, Ostracoda	MK604921	MK604922
Daphnia magna	Arthropoda, Crustacea, Branchiopoda	KZS04090.1	KZS18364.1
Daphnia pulex	Arthropoda, Crustacea, Branchiopoda	EFX74629.1	EFX67118.1
Litopenaeus vannamei	Arthropoda, Crustacea, Malacostraca	ACG60513.1	ACR23316.1
Macrobrachium nipponense	Arthropoda, Crustacea, Malacostraca	AHL24866.1	ANV82809.1
Fenneropenaeus chinensis	Arthropoda, Crustacea, Malacostraca	ABB85237.1	ABB86961.1
Hyalella azteca	Arthropoda, Crustacea, Malacostraca	XP_018024668.1	XP_018019404.1
Penaeus vannamei	Arthropoda, Crustacea, Malacostraca	ROT65546.1	XP_027232902.1
Aedes aegypti	Arthropoda, Hexapoda, Insecta	XP_001656234.1	XP_011493259.1
Anopheles gambiae	Arthropoda, Hexapoda, Insecta	AEE44123.1	XP_319210.4
Anopheles darlingi	Arthropoda, Hexapoda, Insecta	ETN60412.1	ETN63089.1
Aedes albopictus	Arthropoda, Hexapoda, Insecta	XP_019932985.1	KXJ76018.1
Harpegnathos saltator	Arthropoda, Hexapoda, Insecta	XP_011135477.2	XP_011137428.1
Folsomia candida	Arthropoda, Hexapoda, Collembola	XP_021962269.1	XP_021948842.1
Parasteatoda tepidariorum	Arthropoda, Chelicerata, Arachnida	XP_015915002.1	XP_015911244.1
Limulus polyphemus	Arthropoda, Chelicerata, Arachnida	XP_022246826.1	XP_013774138.1
Crassostrea virginica	Mollusca, Bivalvia, Pteriomorphia	XP_022321263.1	XP_022328431.1

sec. The PCR products were cloned into the pMD19-T Simple Vector and sequenced as earlier described.

Cloning of the full-length genomic sequences of DsChi and DsNAG

The design of the PCR primers was based on cDNA sequences obtained from RACE. Genomic DNA extracts of *D. sinensis* were used as templates. The PCR cycling conditions were: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 66 °C for 2 min, and 68 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were gel-purified and cloned into the pMD19-T simple vector. The resultant recombinant clones were screened using PCR and Sanger sequencing.

Bioinformatics analyses of chitinase and NAG sequences

Primary sequences obtained were analyzed using Basic Local Alignment Search Tool (BLAST). Open reading frames (ORFs) were predicted using the ORF finder. The physical and chemical properties of the DpChk1-deduced protein were predicted with ProtParam software, while the conserved domains of each protein were analyzed using Conserved Domains online software. Clustal W software was employed for multisequence alignment. Phylogenetic trees were constructed using maximum likelihood method in the Molecular Evolutionary Genetics Analysis (MEGA 5.1) software, with 1000 replications. The GenBank accession numbers for all sequences used to construct phylogenetic trees are shown in Table 2.

Expression of DsChi and DsNAG mRNAs during growth of D. sinensis

Newly-hatched ostracod neonates (n = 100) were seeded in a 100 mL beaker containing 100 mL M4 medium supplemented with 1 mL *S. subspicatus* (1×10^6 cells/mL). Triplicate samples were drawn from the culture medium at five time points (0, 7, 14, 21, and 28 days). From the day 1 to day 28, the organism showed apparent changes in body length (between 0.2 and 1.5 mm). To determine the inhibitory effect of juvenile hormone analog fenoxycarb on molting, 1 ppm of fenoxycarb was added to the 28-day old *D. sinensis* culture medium. Levels of expression of *DsChi* and *DsNAG* mRNAs were determined with qRT-PCR using the Chi-F–Ch-R and NAG-F–NAG-R primer pairs, respectively (Table 1). The mRNA expression level of β-actin was amplified as an internal control, with actin-F and actin-R as primers (Table 1). Amplification was performed using the Line Gene 9600 Plus system and the SYBR Premix Ex Taq II (TliRNaseH Plus). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (17).

Statistical analysis

Numerical data are expressed as mean ± SEM. Groups were compared using Student *t*-test. Differences in gene expression were compared using Bonferroni correction. Time-series expression data were analyzed using STEM (1.3.11) (18). The SPSS version 21.0 was used for all statistical analyses. Values of $p < 0.05$ were considered statistically significant.

Results

Results of cloning and sequencing of DsChi and DsNAG genes

The *DsChi* cDNA sequence included a coding region of 1,818 bp, a 5′- untranslated region (UTR) of 174 bp, and 3′-UTR of 111 bp (Figure 1). The *DsNAG* cDNA sequence comprised a coding region of 1,089 bp, a 5′-UTR of 153 bp, and 3′-UTR of 339 bp (Figure 2).

Genomic structures of Dschi and DsNAG genes

After alignment of the genomic sequences with cDNA sequences nine introns in the *DsChi* gene (Figure 3A) and five introns in the *DsNAG* gene were identified

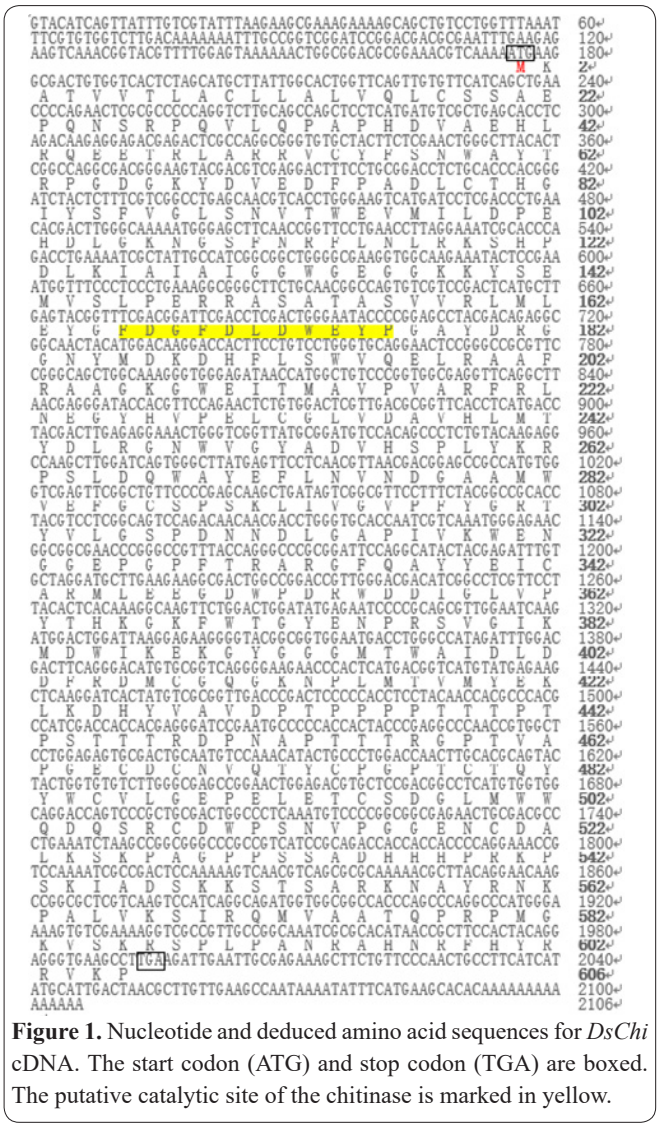

Figure 1. Nucleotide and deduced amino acid sequences for *DsChi* cDNA. The start codon (ATG) and stop codon (TGA) are boxed. The putative catalytic site of the chitinase is marked in yellow.

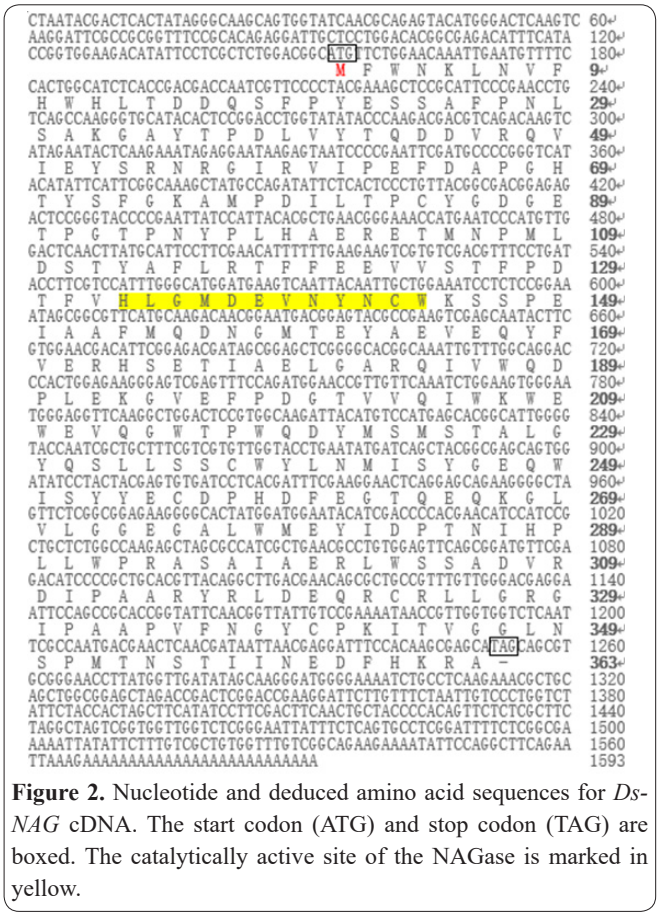

Figure 2. Nucleotide and deduced amino acid sequences for *DsNAG* cDNA. The start codon (ATG) and stop codon (TAG) are boxed. The catalytically active site of the NAGase is marked in yellow.

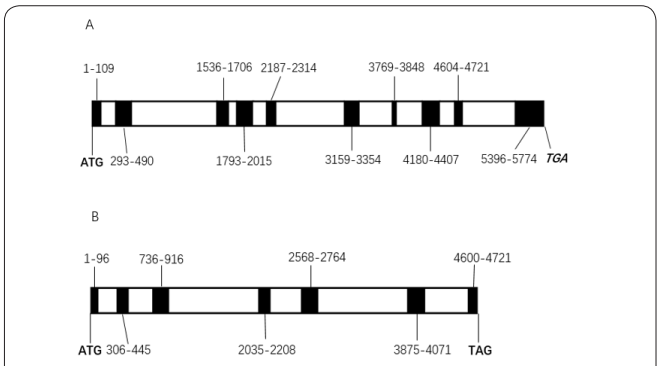

Figure 3. Schematic representations of the genomic structures of the two genes. (A): Genomic structure of *Dschi*; and (B): Genomic structure of *DsNAG*. Black boxes represent exons, while white boxes represent introns. The numbers indicate the start and end positions of exons relative to the translation start site of each gene.

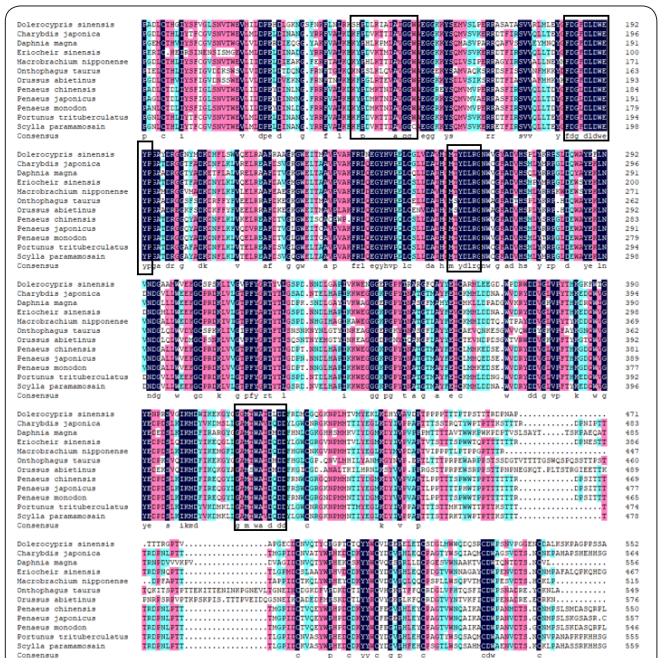

Figure 4. Multiple alignment of chitinase protein sequences from different arthropods. Color shadows indicate homology levels (black = 100 %, pink ≥ 75 %, blue ≥ 50 %). Four conserved motifs are indicated in the box. All sequences are available from GenBank with accession numbers indicated in parentheses: *Penaeus japonicas* (BAA12287.1), *Charybdis japonica* (AFF59213.1), *Daphnia magna* (KZS04090.1), *Scylla paramamosain* (AWU46589.1), *Macrobrachium nipponense* (AHL24866.1), *Eriocheir sinensis* (AKP18000.1), *Onthophagus taurus* (XP_022902719.1), *Orussus abietinus* (XP_012280096.1), *Penaeus monodon* (XP_012280096.1), *Penaeus chinensis* (ABB85237.1), *Portunus trituberculatus* (AKE49855.1), and *Dolerocypris sinensis* (MK604921 obtained in this study).

(Figure 3B).

Results of comparative and phylogenetic analyses of the DsChi and DsNAG genes in D. sinensis

When compared with protein sequences available in GenBank, chitinase from *D. sinensis* was most closely related to that of *Macrobrachium nipponense* (61 % homology), followed by *Litopenaeus vannamei* (56.1 %) and *Fenneropenaeus chinensis* (55.5 %). The NAG of *D. sinensis* was most closely related to that of *Limulus polyphemus* (55.6 % homology), followed by *Parasteatoda tepidariorum* (54.55 %) and *Folsomia candida*

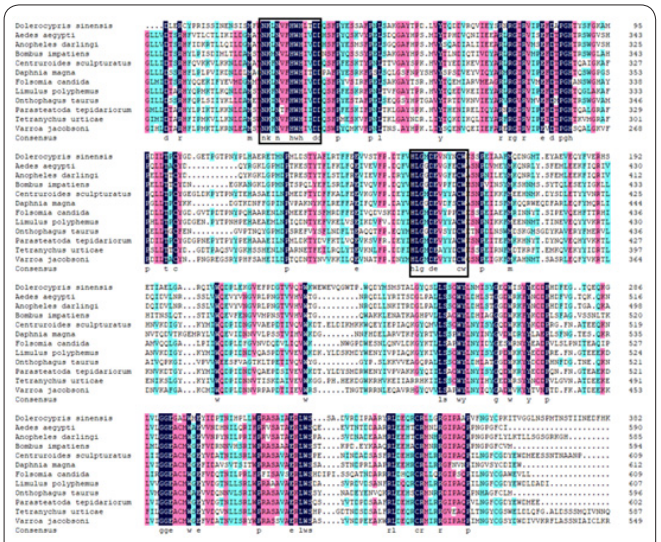

Figure 5. Multiple alignments of NAG protein sequences from different arthropods. Color shadows indicate homology levels (black = 100 %, pink ≥ 75 %, blue ≥ 50 %). Two conserved motifs are indicated in the box. All sequences are available at GenBank with accession numbers indicated in parentheses: *Onthophagus taurus* (XP_022921143.1), *D. magna* (KZS16343.1), *Aedes aegypti* (XP_001650049.1), *Limulus polyphemus* (XP_013774138.1), *Bombus impatiens* (XP_003488866.1), *Folsomia candida* (XP_021948842.1), *Varroa jacobsoni* (XP_022709546.1), *Parasteatoda tepidariorum* (XP_015911244.1), *Tetranychus urticae* (XP_015781043.1), *Anopheles darlingi* (ETN63089.1), *Centruroides sculpturatus* (XP_023219978.1) and *Dolerocypris sinensis* (MK604922 obtained in this study).

(53.8 %). An alignment of known chitinase sequences from Arthropods showed four highly conserved regions: KxxxxxGGW, FDGxDLDWEYP, MxYDxxG, and GxxxWxxDxDD, where x represents variable amino acid residues (Figure 4). In addition, two highly conserved regions were observed in the NAG proteins from Arthropods: NKxNxxHWHxxDD and HxGMDEVxxxCW (Figure 5).

Results of phylogenetic analysis

Based on phylogenetic analysis of known chitinases from crustaceans and insects, *D. sinensis* chitinase tightly clustered in the same branch with chitinases from species within the Malacostraca class: *M. nipponense*, *Litopenaeus vannamei*, and *Fenneropenaeus chinensis* with fairly strong bootstrap support of 89 % (Figure 6). In contrast, the *D. sinensis* NAG clustered with NAG from *F. candida* in the same branch with weak bootstrap support of 56 % (Figure 7). However, in both phylogenetic trees, not all species were grouped according to their taxonomic classification; species from the same sub-phylum were separated into two or more different clades, separated by clades comprising species form other subphyla.

Predicted general chemical properties of DsChi and DsNAG proteins

The results of bioinformatics showed that *DsChi* gene encodes a protein of 606 amino acids with predicted molecular mass of 68 kDa and pI of 6.96. The deduced protein structure revealed typical structural features of chitinase such as presence of a signal peptide, a GH18 catalytic domain, an S/T-enriched junction region, and a chitin-binding site. The *DsNAG* gene encoded a protein of 366 amino acids, with predicted molecular mass of 42 kDa and pI of 4.62. The deduced protein structure had only a GH20 catalytic region.

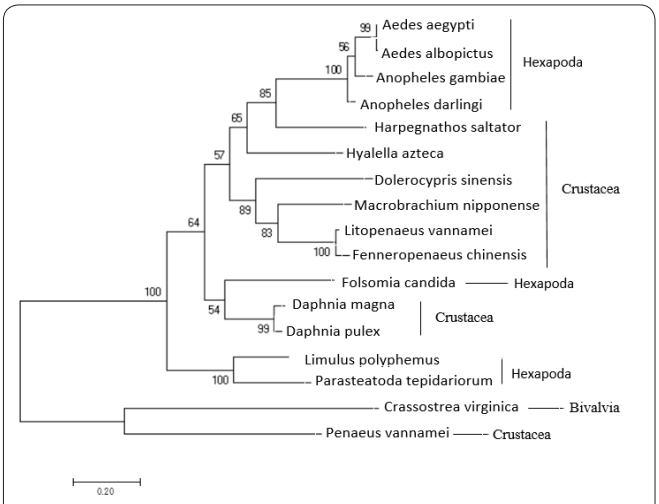

Figure 6. Phylogenetic tree constructed using maximum likelihood method and chitinase protein sequences from 17 species. Numbers at branches indicate the bootstrap proportion (%), calculated with 1000 replicates. GenBank accession numbers for all sequences and the taxonomic classifications for all species referenced are shown in Table 2.

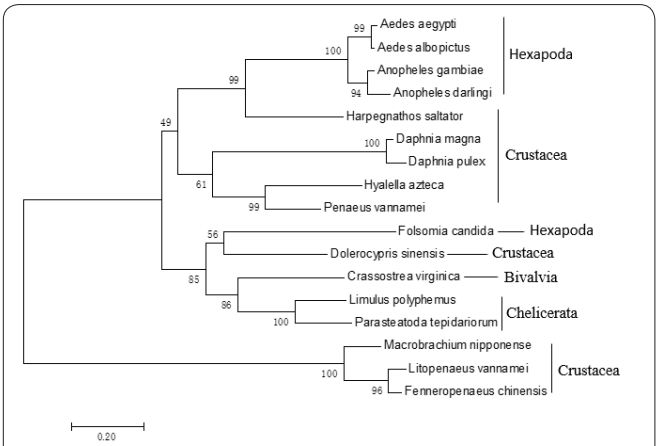

Figure 7. Phylogenetic tree constructed using maximum likelihood method and protein sequences for NAG from 17 species. The numbers at branches indicate the bootstrap proportion (%), calculated with 1000 replicates. GenBank accession numbers for all sequences and taxonomic classifications for all species referenced are shown in Table 2.

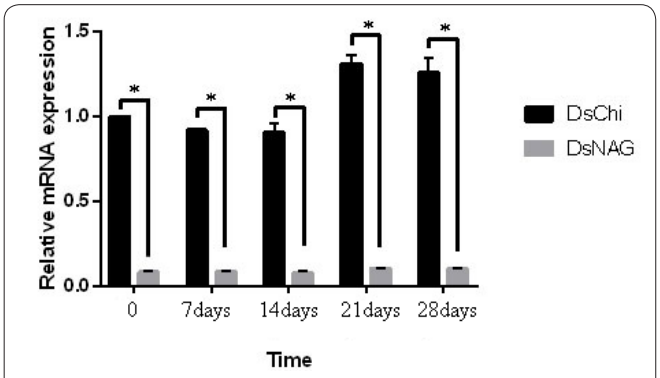

Figure 8. Relative expressions of *DsChi* and *DsNAG* mRNAs at various stages of growth of *D. sinensis*. $^{*}p < 0.05$, as compared using Bonferroni correction.

Expressions of DsChi and DsNAG mRNAs throughout the growth period of D. sinensis

The level of expression of *DsChi* mRNA was significantly higher than that of *DsNAG* throughout the period of growth of *D. sinensis* ($p < 0.05$). There were no significant differences in mRNA expression levels among the different time points for both genes ($p < 0.05$; Figure 8).

Effect of fenoxycarb on the expressions of DsChi and DsNAG mRNAs

Treatment of *D. sinensis* cells with fenoxycarb significantly downregulated the expression of *DsChi* at 3, 6, 12, and 72 h ($p < 0.05$), but did not significantly alter the expression of *DsChi* at 18, 24, or 48 h ($p > 0.05$; Figure 9A). However, treatment of *D. sinensis* cells with fenoxycarb significantly downregulated the expression of *DsNAG* throughout the growth period ($p < 0.05$). The inhibitory effect of fenoxycarb on *DsNAG* expression was maximum 6 h post-treatment (Figure 9B).

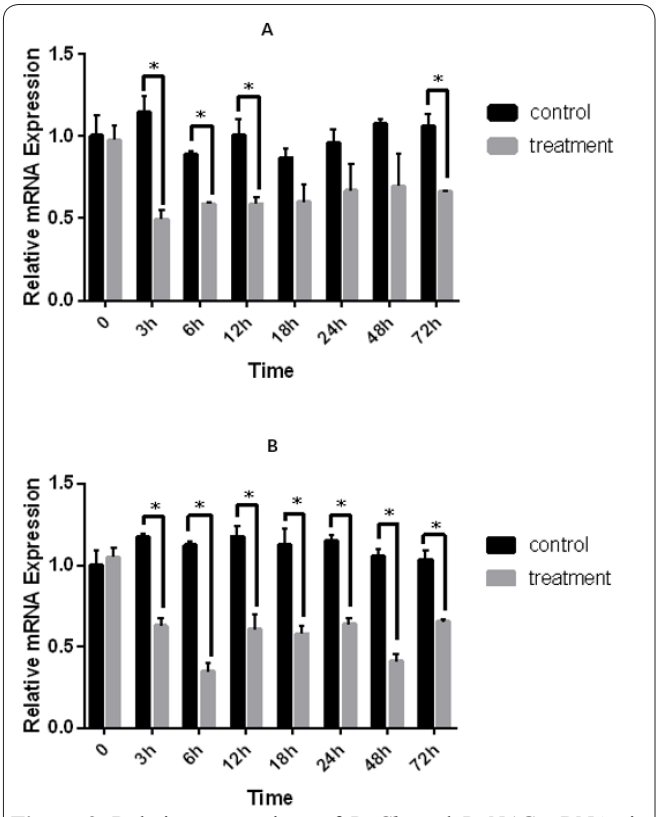

Figure 9. Relative expressions of *DsChi* and *DsNAG* mRNAs in culture medium after exposure to fenoxycarb. (A): Relative expression of *DsChi* mRNA; and (B): Relative expression of *DsNAG* mRNA. $^{*}p < 0.05$, as compared using Bonferroni correction.

Discussion

Chitinase and NAG play key roles in the growth of crustaceans and insects. Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. They are found in organisms that either need to reshape their own chitin or dissolve and digest the chitin of fungi or animals.

This study represents the first attempt to clone the full-length cDNA and genomic DNA sequences encoding chitinase and NAG proteins in *D. sinensis*, and to describe their basic molecular properties, while elucidating their phylogenetic relationships with known chitinolytic enzymes from other Arthropoda species. The study also described their expression pattern at various stages of growth of the organism, and the genetic and transcriptional characteristics of chitinase and NAG in a species within the Ostracoda class. The results obtained suggest that the *DsChi*-encoded protein possesses typical characteristics of known chitinases in insects and crustaceans, with four highly conserved domains: KxxxxxGGW, FDGxDLDWEYP, MxYDxxG, and GxxxWxxDxDD. These four domains are believed to form the catalytic site and maintain the enzyme's function (19, 20).

The results of studies on site-directed mutagenesis of insect chitinases suggest that one of these domains (FDGxDLDWEYP) plays an important role in chitinolytic activity (21). Similarly, *DsNAG* protein also possesses characteristics typical of known NAGs in insects and crustaceans, as well as two conserved domains: NKxNxxHWHxxDD and HxGMDEVxxxCW. These two domains exist in all members of the GH20 family which includes all NAGs (22). The results of phylogenetic analysis suggest that chitinase and NAG may have evolved independently in *D. sinensis*. The results of qRT-PCR revealed that *DsChi* and *DsNAG* are expressed in *D. sinensis* from birth until maturity. It is likely that the expression levels of these proteins remain fairly stable throughout the period of growth. This pattern of chitinase expression differs from that observed in daphnia, which expresses chitinase at very low level at birth, followed by a rapid increase throughout growth until the point of maturity, when expression decreases sharply (23). This difference may be due to the fact that ostracods (including *D. sinensis*) have longer life cycle, slower growth, and less frequent molting than daphnia. Ostracods and daphnia are dominant, and usually coexist harmoniously in aquatic habitats (24) . The finding that chitinase expression changes considerably over time in daphnia, but remains relatively stable in ostracods suggests that the fluctuation in chitinase activity in aquatic environments most likely depends on population-specific characteristics for daphnia, when compared with ostracods and other species.

In this study, the expression of *DsChi* was significantly higher than that of *DsNAG* throughout the period of growth, an indication that *DsChi* may be more actively involved in the growth of *D. sinensis*. Studies have shown that chitinases play a dominant role in the degradation of chitin (25). Treatment of *D. sinensis* cells with the juvenile hormone analog fenoxycarb significantly downregulated the expressions of *DsChi* and *DsNAG* throughout the growth period. These results are in agreement with those reported for *Bombyx mori* (26). There are reports that environmental pollutants containing PAHs inhibit NAG expression in *Penaeus aztecus* (27). These results suggest that environmental pollutants such as fenoxycarb and PAHs may be harmful to aquatic life and environments.

Industrial and agricultural activities impact negatively on the aquatic environment. The results of this study are in agreement with those previously reported, and suggest that many pollutants may inhibit the activity of chitinolytic enzymes in aquatic organisms. Inhibition of these enzymes is expected to prolong the cycle time required for molting, leading to immature deve-

lopment and even death of crustaceans (28). The level of contamination in an aquatic environment correlates with changes in expression of chitin hydrolase mRNA or protein in outer epidermal tissue (29). Future studies should focus on whether *DsChi* expression can be used to monitor the dynamics of ostracod population shift from one aquatic environment to another, or contamination levels of environmental pollutants. The availability of complete gene sequences should facilitate the development of antibodies for immunodetection of *DsChi* and *DsNAG* proteins.

The results obtained in this study have shown that the protein products of *DsChi* and *DsNAG* possess remarkable biochemical properties characteristic of chitinase and NAG, respectively.

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Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Shaonan Li; Gong Cheng, Shaonan Li collected and analysed the data; Gong Cheng wrote the text and all authors have read and approved the text prior to publication.

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