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Molecular characterization of the gene checkpoint homolog 1 in *Daphnia carinata* during different reproductive phases

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ABSTRACT. Full-length cDNA of the gene checkpoint homolog 1 (*Chk1*) was cloned from *Daphnia carinata* and designated *DcarChk1*. *DcarChk1* cDNA was 1817 bp in length and encoded a 497-amino acid polypeptide. Phylogenetic analyses revealed that *DcarChk1* was most closely related to *Chk1* of *Daphnia pulex*, followed by homologous genes of insects. Expression of *DcarChk1* was higher in adult *Daphnia* than in larvae, and significantly higher in males than females, as determined by real-time polymerase chain reaction analysis. Using whole-mount *in situ* hybridization techniques, *DcarChk1* in parthenogenetic females was found to be expressed mainly on the head surface, capillus, and carapace valve edge. In contrast, in sexual females, *DcarChk1* was expressed mainly in the joint of the second antenna, and in the thoracic limbs and capillus. These results suggest that *DcarChk1* plays a significant role in both the growth and development, as well as in regulating reproductive plasticity, in *D. carinata*.

Key words: *Daphnia carinata*; Cloning; Expression; Whole-mount *in situ* hybridization; Checkpoint homolog 1

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INTRODUCTION

Checkpoint homolog 1 (*Chk1*) is a type of serine/threonine-protein kinase gene, and is involved in regulating the cell cycle checkpoint by DNA damage (Merry et al., 2010). It was initially discovered and identified as a cell cycle detection gene in fission yeast (Walworth et al., 1993). Subsequently, homologs have been found in mammals, insects, birds, amphibians, budding yeast, and other eukaryotes; all have a highly conserved structure and function (Chen and Sanchez, 2004). *Chk1* is the downstream target of the ataxia telangiectasia-mutated (*ATR*) and ataxia telangiectasia-mutated gene (*ATM*) (Liu et al., 2010), which are sensors in the signal transduction pathway of the DNA damage checkpoint (Li et al., 2006). When DNA is damaged, *ATR* and *ATM* are activated and regulate different signal factors to end the cell cycle in G2/M (Wang et al., 2006a; Pan et al., 2009). *Chk1* is the core molecular check for damaged DNA in G2/M. As a transducin, it can transmit amplifying signal to the effector Cdc25c, which enacts phosphorylation of Cdc25c to repair damaged DNA (Rouse and Jackson, 2002; Ling et al., 2010). Hence, when expression levels of *Chk1* decrease, damaged DNA is no longer detected or repaired efficiently, which leads to cell apoptosis (Sørensen et al., 2005; Syljuåsen et al., 2005; Bolderson et al., 2009; Carrassa et al., 2009). Clinical studies have found that *Chk1* is expressed in a variety of tumor cells (Bartek and Lukas, 2003). Moreover, other studies have shown that RNA interference silences *Chk1* and reduces its expression, resulting in limited reparation of tumor cells and increased mortality. A robust understanding of the expression levels of *Chk1* will, therefore, be particularly useful for the advancement of cancer therapies (Slupianek et al., 2002; Zhang et al., 2008; Chen et al., 2009).

Daphnia carinata is a common microzooplankton which, because of its high protein content, is used as a high quality feed in commercial fisheries (Yang, 1994). It is a small organism, is fast breeding and readily cultivated, and is sensitive to environmental variation; these characteristics make it ideal as a biological indicator of water quality in environmental monitoring studies (Qi et al., 1991). Compared to other invertebrates, cladocerans such as *Daphnia* spp have a unique life history that involves facultative parthenogenesis. Under suitable conditions, *Daphnia* spp reproduce parthenogenetically, but as conditions deteriorate, bisexual reproduction occurs. Eggs produced withstand the harsh environment, and hatch when conditions improve (Hebert, 1978). This reproductive plasticity is an adaptive advantage in variable environments, and ensures population persistence (Stross and Hill, 1965). While the ecology of this reproductive plasticity has received much attention (Martinez-Jeronimo et al., 1994; Khmeleva et al., 1995; Cao et al., 2001), knowledge of regulatory molecular mechanisms is lacking. In this study, we use molecular tools to explore the potential role of *Chk1* in regulating growth and reproduction in *D. carinata*. The data presented on the expression pattern and function of *DcarChk1* in *D. carinata* provide a basis for understanding the molecular mechanisms driving growth and reproductive plasticity in cladocerans.

MATERIAL AND METHODS

Sample preparation

D. carinata were obtained from the College of Life Sciences of Ningbo University. Healthy parthenogenetic individuals were light cultured at 25°C and fed with cladoceran nutrient solution for 2 to 3 weeks. The solution contained 1.5 g cow dung, 2 g vegetables, 20 g fertile soil, and 1 L water. The vegetables were shredded and boiled for 5 to 6 min, allowed to cool to 60° to 70°C, and then

the dung and soil were added and the solution filtered. Parthenogenetic and sexual females (N = 150) were isolated, frozen in liquid nitrogen, and stored at -80°C for RNA extraction. A further 30 individuals were fixed in 4% paraformaldehyde at 4°C overnight, after which the paraformaldehyde was replaced with 100% methanol, and specimens were stored at -20°C for whole-mount *in situ* hybridization.

Primer design

Forward (F1) and reverse (R1) primers were designed to amplify the conserved region of *DcarChk1* from sequence of the *Chk1* homolog of *Daphnia pulex* (GenBank accession No. AGN95867; Table 1). Based on this partial sequence, two pairs of gene-rapid amplification of cDNA ends (RACE) primers (F2, R2; Table 1) were designed for RACE-polymerase chain reaction (PCR) in order to clone the whole gene sequence. Full-length *DcarChk1* was used to design a pair of primers for quantitative PCR (qPCR), F3, and R3, targeted to the open reading frame (ORF) sequences of the gene (Table 1). Specific primers to amplify the L8 ribosomal gene (L8-F, L8-R; Table 1) were designed according to the conserved sequence. For whole-mount *in situ* hybridization, upstream (F4) and downstream (R4) primers in the ORF of *DcarChk1* were designed for the preparation of riboprobe (Table 1). All primers were purchased commercially (Sangon, China).

Table 1. Primers used in the study.

Primer	Sequence (5'-3')	Purpose
F1	TGGGTGAAGGAGCTTTTGGAGAGG	Standard PCR
R1	ATCCCAAGGCAATTCTCCGGC	
F2	CCAATGCTGCTGAAACGGTCAAGA	RACE-PCR
R2	GGGGCATTCCCACATCAGGCTCTAT	
UPM	CTAATACGACTCACTATAGGGCAAGC	Kit primers
	AGTGGTATCAACGCAGAGT	
	CTAATACGACTCACTATAGGGC	
F3	AGGCGCAGTGTTTAGCTTCT	Real-Time PCR
R3	CCGGGGGTGTGTATTTTCCA	
L8-F	CCGAGGTCCTATTCCATCAT	L8 reference gene
L8-R	TCGGATGTCTGTCTTCGGC	
F4	TTGGGTTTCTTCTCCGGTTC	Riboprobe
R4	TGGCCATTGACCATGTTACT	

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted using an RNA extraction kit (Axygen, USA) according to the manufacturer protocol. The degree of RNA degradation was determined by agarose gel electrophoresis. The concentration and optical density (OD) values of samples were measured using a nucleic acid protein detector. Clear bands with no tail and OD values between 1.8 and 2.0 ng/μL indicated that RNA extraction was successful. For standard and qPCR, first-strand cDNA was synthesized using a reverse transcription kit (TaKaRa, Japan). For RACE-PCR, a SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used. Five prime- and 3'-RACE-ready cDNA templates were prepared according to the manufacturer instructions. Synthesized DNA was stored at -20°C.

Cloning of partial- and full-length *DcarChk1*

Using first-strand cDNA of adult parthenogenetic *Daphnia* as a template and a Premix Ex Taq™ HS Kit (TaKaRa, Japan), *DcarChk1* partial sequence was generated. Amplification by PCR

was performed in a total volume of 25 µL under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C 30 s, and 72°C for 1 min; and a final extension of 72°C for 10 min, followed by storage at 4°C. The PCR products were separated using 1.2% agarose (w/v) gel electrophoresis, and the appropriate bands were sent to Major Biotechnology Co., Ltd (Shanghai) for sequencing. BLAST was used to compare products against the NCBI database; if similarity was greater than 80% and gene names were consistent, then partial cDNA sequence of *DcarChk1* was confirmed. Full-length cDNA of *DcarChk1* was generated by further RACE-PCR amplification, under the same PCR conditions described above. The melting temperatures of 5'- and 3'-RACE primers were 58° and 63°C, respectively. Amplification products were separated using agarose gel electrophoresis and the appropriate bands were cloned and sequenced by Major Biotechnology Co., Ltd.

Bioinformatics analysis of *DcarChk1*

Sequencing results for the 3'- and 5'-RACE PCR products were screened against the NCBI database, splicing the right 5' and 3' sequences to confirm the full-length cDNA of *DcarChk1*. For comparative analyses of gene and protein sequences, the NCBI BlastX tool (http://www.ncbi.nlm.nih.gov) was used. For analysis of ORFs and predicted amino acid (aa) sequences, the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used. The isoelectric point (pI) and molecular weight (MW) of proteins were calculated using the EXPASY database (http://web.expasy.org/cgi-bin/protscale/protscale.pl). The SignalP software (http://www.cbs.dtu.dk/services/signalp) was used for signal peptide identification. Characteristics of the structural domain were analyzed using the Simple Modular Architecture Research Tool SMART (http://www.smart.emblheidelberg.de). The BLAST feature of the NCBI database was used to extract protein names, sequences, and aa numbers for the phylogenetic analysis. ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2) was used for multiple sequence alignment of *Chk1* genes, and the MEGA 4.0 software was used to construct the phylogenetic tree using the neighbor-joining method with a bootstrap value of 1000.

Real-time qPCR analysis

The concentration of cDNA templates in different growth and reproductive phases, and qPCR target gene expression was normalized against that of the L8 ribosomal gene. Reactions contained 12.5 µL SYBR Premix Ex Taq II, 1 µL cDNA template, 0.5 µL primer F3 (10 µM), 0.5 µL primer R3 (10 µM), and ddH_2O to form a final volume of 25 µL. Conditions for PCR were 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 30 s. Samples were assayed in triplicate, and melting and amplification curves were constructed from the results. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). One-way ANOVA was used to assess significance differences in *DcarChk1* expression among different reproductive phases; analyses were undertaken using the SPSS software.

Riboprobe preparation

Using the upstream (F4) and downstream (R4) primers described above (Table 1), PCR amplification was used to prepare *in situ* hybridization probes, with a probe length of 499 bp, and a melting temperature of 57°C. Products were separated by electrophoresis on 1.2% agarose (w/v) gel extracted using a QIAquick Gel Extraction kit. Purified products were ligated into the

pGEM-T vector (Promega, USA) using T4 DNA ligase (Promega, USA) and then transformed into *Escherichia coli* strain DH5α. Following blue-white screening, positive clone bacterium liquid was sent to BioSune Biotechnology Co. Ltd. (Shanghai) for sequencing. Bacterium solutions positive for target inserts were cultured and the plasmid extracted. According to the multiple cloning site of PGEM-T Vector and the restriction enzyme cutting site of the *DcarChk1* gene probe sequence, the restriction enzymes *Nco*I and *Spe*I were used for linearization. Digested products were gel-purified and used as sense and antisense probes of *DcarChk1* template. Transcription systems (20 µL) were set up in 1.5-mL centrifuge tubes containing: 4 µL 5X transcription buffer; 2 µL DTT (100 mM); 0.5 µL RNase inhibitor (40 U); 8 µL linear DNA (<1 µg); 2 µL 10X DIG labeling mixture; 1 µL SP6 or T7 RNA polymerase (20 U/µL); and 2.5 µL 0.1% diethylpyrocarbonate (DEPC) Millipore H_2O. Reactions were mixed and incubated at 37°C for 2 h. Then, 1 µL DNase (RNase-free) was added and incubation was continued for 15 min. A 1/9 volume of 5 M LiCl and 2 volumes of absolute ethanol were added and incubation continued at -20°C overnight. Mixtures were centrifuged at 12,000 rpm for 30 min at 4°C and supernatants discarded. Then, RNA pellets were washed twice with 75% ethanol, centrifuged at 13,000 rpm for 5 min, and thoroughly dried to remove residual ethanol. Lastly, RNA sediment was re-suspended in 30 µL DEPC water, and 1.5 µL RNA inhibitor (20 U) was added. Aliquots of RNA solutions (1 µL) were added and electrophoresed; concentrations were measured while remaining RNA probes were stored at -20°C.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out according to previously published methods (Thisse and Thisse, 2008; Zhang and Liu, 2013) with some modifications. Briefly, on the first day, specimens stored at -20°C were rehydrated gradually with methanol-PBST and digested with proteinase K (10 µg/mL). Sexually reproducing and parthenogenetic individuals were digested at 37°C for 15 and 12 min, respectively. Following pre-hybridization at 67°C for 2.5 h, 100 µL RNA probe diluted 1:100 was added and incubation continued at 67°C overnight. On the second day, probes were aspirated and residuum washed gradually with hybridization 2X SSC and 0.2X SSC-MABT. Specimens were blocked for approximately 2 h at room temperature with slow shaking in MAB block solution. Then, anti-DIG antibody (diluted 1:5000; Roche, USA) was added and specimens incubated at 4°C for 13 h. On the third day, antibody solution was discarded and specimens were washed briefly in MABT. Then, NBT liquid dye (Roche) and TSM2 were added at a ratio of 1:50 and specimens incubated at room temperature in the dark for approximately 10 min. Finally, individuals were fixed in 4% paraformaldehyde. Hybridization was visualized and recorded using a BX51 fluorescence microscope (Olympus, USA).

RESULTS

Cloning of full-length *DcarChk1* cDNA

Using *D. carinata* template cDNA and gene-specific primers, a 584-bp fragment was obtained by PCR amplification. BLASTx comparison showed high homology to the gene homolog of *D. pulex* from the NCBI database. On this basis, gene-RACE primers were designed, and 5'- and 3'-RACE PCR yielded 1442- and 631-bp fragments, respectively. Fragments were spliced with an intermediate fragment in order to construct full-length (1817 bp) *DcarChk1* cDNA (GenBank accession No. KJ735446; Figure 1).

```
   1  atggggacatttgagtcaaaatgccacctgttctggtaatgaaatcaattgtgtgat
  58  ttctgctacgtgagttcaatccgtgggtgactattcatttaacggtgttaaaaagccacc
 118  gaaaagacgcggaaaaagtggtaatacgtgttagcttgtcgtctttatctgtgtcttaa
 178  cgggttgtcaccttcgcacctccttgtgatctgttgtaaagaaagtatttatcaccaatt
 238  atggatgaaaaaagtaaaaagtatgaaggaaaaaacagtgtaattgaatttgtagaaggt
   1  M  D  E  K  S  K  K  Y  E  G  K  N  S  V  I  E  F  V  E  G
 298  tgggatatgatacagacactgggtgaaggagcgtttggggaggtaaaactactagtcaat
  21  W  D  M  I  Q  T  L  G  E  G  A  F  G  E  V  K  L  L  V  N
 358  gcaaaaactggagaagcagtggcaatgaaagtaattgatttgaagaaacatgccaatgct
  41  A  K  T  G  E  A  V  A  M  K  V  I  D  L  K  K  H  A  N  A
 418  gctgaaacggtcaagaaagaagtttgtgttcacagaatgttaaatgaccctcatgtcatt
  61  A  E  T  V  K  K  E  V  C  V  H  R  M  L  N  D  P  H  V  I
 478  cggttttatggcagaagggaaaatggcaattttgaattcatttttttggaatatgcaagt
  81  R  F  Y  G  R  R  E  N  G  N  F  E  F  I  F  L  E  Y  A  S
 538  ggtggagagctctttgacagaatagagcctgatgtgggaatgccccaaatggaagcccag
 101  G  G  E  L  F  D  R  I  E  P  D  V  G  M  P  Q  M  E  A  Q
 598  cgttatttcaaacaattgattgctggagtaaattacttgcatagcagaggagttgctcat
 121  R  Y  F  K  Q  L  I  A  G  V  N  Y  L  H  S  R  G  V  A  H
 658  agagacattaaaccagaaaatttgcttttggatgcaaatgataatttgaaaatatctgat
 141  R  D  I  K  P  E  N  L  L  L  D  A  N  D  N  L  K  I  S  D
 718  tttggaatggcgacaatttttcgatttcaaggtcgagaaagacttctggataaacgttgt
 161  F  G  M  A  T  I  F  R  F  Q  G  R  E  R  L  L  D  K  R  C
 778  ggaactttgccttacatagctcctgaagtgctttgtcgcaagtacgcagcagaaccggct
 181  G  T  L  P  Y  I  A  P  E  V  L  C  R  K  Y  A  A  E  P  A
 838  gatatttggtcttgtggcgtcgtccttgttgccatgttagctggagaattaccttgggat
 201  D  I  W  S  C  G  V  V  L  V  A  M  L  A  G  E  L  P  W  D
 898  gtcccttctaatgattgtcctctctatacatcatggaaagaatgtcagataacgcgattg
 221  V  P  S  N  D  C  P  L  Y  T  S  W  K  E  C  Q  I  T  R  L
 958  ccatggacaaagattgatacacttgctttgtctttactgcgaaaagtgctgatgcctctg
 241  P  W  T  K  I  D  T  L  A  L  S  L  L  R  K  V  L  M  P  L
1018  ccagggaagcgttataccattcaacaaataactaaccatcagtggtttcaaaaaatattt
 261  P  G  K  R  Y  T  I  Q  Q  I  T  N  H  Q  W  F  Q  K  I  F
1078  aaagtttcaagtacatctcttagagcagaagaaaacacacctgtatctaaacggatatgt
 281  K  V  S  S  T  S  L  R  A  E  E  N  T  P  V  S  K  R  I  C
1138  tctgacgccgtggacgccggtttgccaccgtcttcatcggacgccagccgcctgtcatac
 301  S  D  A  V  D  A  G  L  P  P  S  S  S  D  A  S  R  L  S  Y
1198  tctcagcctggcttgggtttcttctccggttcccaaccggtacaccaaaacgacaacaat
 321  S  Q  P  G  L  G  F  F  S  G  S  Q  P  V  H  Q  N  D  N  N
1258  gatgatgaagagccaaataatcttccaggcgcagtgtttagcttctcccaacccgctcat
 341  D  D  E  E  P  N  N  L  P  G  A  V  F  S  F  S  Q  P  A  H
1318  attgacggtatgctacttaactcgcagttgaacacgcagaccgcttcaggttcaagcatg
 361  I  D  G  M  L  L  N  S  Q  L  N  T  Q  T  A  S  G  S  S  M
1378  agttctccactgcaaaggctcgttaaaaggatgactcgtttggtggctaaagtcagctgt
 381  S  S  P  L  Q  R  L  V  K  R  M  T  R  L  V  A  K  V  S  C
1438  gaagaagcaatcaagcatttgagccaacaactaatcaaacttggctacacttggaaaata
 401  E  E  A  I  K  H  L  S  Q  Q  L  I  K  L  G  Y  T  W  K  I
1498  cacacccccggagtggttactatatcaactcaagatcgacgcaaaatgcaacttgttttc
 421  H  T  P  G  V  V  T  I  S  T  Q  D  R  R  K  M  Q  L  V  F
1558  aaagcaacagtttatgatatgcagacaatggtgttgctcgactttcggctgtcgagaggc
 441  K  A  T  V  Y  D  M  Q  T  M  V  L  L  D  F  R  L  S  R  G
1618  tgtggactggatttcaaaagacatttcctagccattaaacataagttagctgatattttg
 461  C  G  L  D  F  K  R  H  F  L  A  I  K  H  K  L  A  D  I  L
1678  tgttcagcaccagtaacatggtcaatggccactgctactaatagcattcctgacaacc
 481  C  S  A  P  V  T  W  S  M  A  T  A  T  N  S  I  P  *
1738  tttttaactatcttgttttataaatgttgaattacacagagtgttttgtagcaaaaaaaa
1798  aaaaaaaaaaaaaaaaaaaa
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Figure 1. Full-length *DcarChk1* and corresponding amino acid sequence. Initiation (ATG) and termination (TGA) codon are highlighted in bold and boxed. S_TKc domain is shaded in yellow.

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Analysis of *DcarChk1* sequence and of the encoding protein structure

DcarChk1 cDNA contained a 1494-bp ORF; 237-bp 5'-untranslated region (UTR); 86-bp 3'-UTR; and a 3'-polyA tail. The protein encoded by the ORF was 497 aa in length, and had a calculated MW of 55.84 kDa, a theoretical pI of 8.65, and no signal peptide. A catalytic domain with the activity of a serine/threonine-protein kinase (S_TKc domain) was detected at aa positions 21-276 (Figure 1, shaded yellow).

Analysis of *DcarChk1* amino acid homology

Amino acid sequences encoded by *DcarChk1* were compared with those of other *Chk1* homologs in GenBank (accession Nos. are given in Table 2). The highest level of homology was to *Chk1* of *D. pulex* (94.37%). Levels of homology with other species (*Apis dorsata*, *Bombus terrestris*, *Megachile rotundata*, *Anopheles darlingi*, *Acyrthosiphon pisum*, *Metaseiulus occidentalis*, *Saccoglossus kowalevskii*, *Crassostrea gigas*, *Strongylocentrotus purpuratus*, *Ornithorhynchus anatinus*, and *Cavia porcellus*) ranged from 50 to 54% (Figure 2). Upon phylogenetic tree construction, *Chk1* homologs formed two divergent branches (Figure 3). *D. carinata* and *D. pulex* were closely related and clustered together with the hymenopteran insects and arachnids, while other species were more distantly related.

Table 2. Accession numbers of *Chk1* from various species.

Organism	Gene name (definition in NCBI)	Accession No
Daphnia pulex	checkpoint kinase 1	AGN95867
Apis dorsata	PREDICTED: serine/threonine-protein kinase grp-like	XP_006608254
Bombus terrestris	PREDICTED: serine/threonine-protein kinase grp-like	XP_003401057
Megachile rotundata	PREDICTED: serine/threonine-protein kinase grp-like	XP_003705992
Anopheles darlingi	Serine/threonine-protein kinase grp	ETN58880
Acyrthosiphon pisum	PREDICTED: serine/threonine-protein kinase grp-like	XP_001951665
Metaseiulus occidentalis	PREDICTED: serine/threonine-protein kinase Chk1-like	XP_003745318
Crassostrea gigas	Serine/threonine-protein kinase Chk1	EKC39820
Saccoglossus kowalevskii	PREDICTED: serine/threonine-protein kinase Chk1-like	XP_002741282
Strongylocentrotus purpuratus	checkpoint kinase 1	ABK80717
Danio rerio	PREDICTED: serine/threonine-protein kinase Chk1 isoform X1	XP_005161214
Anolis carolinensis	PREDICTED: serine/threonine-protein kinase Chk1-like	XP_003225759
Pseudopodoces humilis	PREDICTED: serine/threonine-protein kinase Chk1	XP_005528500
Cavia porcellus	PREDICTED: serine/threonine-protein kinase Chk1	XP_003472613
Ornithorhynchus anatinus	PREDICTED: serine/threonine-protein kinase Chk1	XP_001513209

PCR analysis of *DcarChk1* expression in different reproductive phases

DcarChk1 expression was detected in both females and males. Levels of expression were significantly higher in males than in females ($P < 0.01$), and in sexual females than in parthenogenetic females (Figure 4). Expression of *DcarChk1* was also found to be higher in adults than in larvae, regardless of the sexual phase of the females (Figure 4).

Cellular localization of DcarChk1 mRNA expression in parthenogenetic and sexual females

DcarChk1 mRNA expression and cellular localization in different reproductive phases of *Daphnia* females were investigated using a digoxin-labeled RNA riboprobe, which bound to cell-

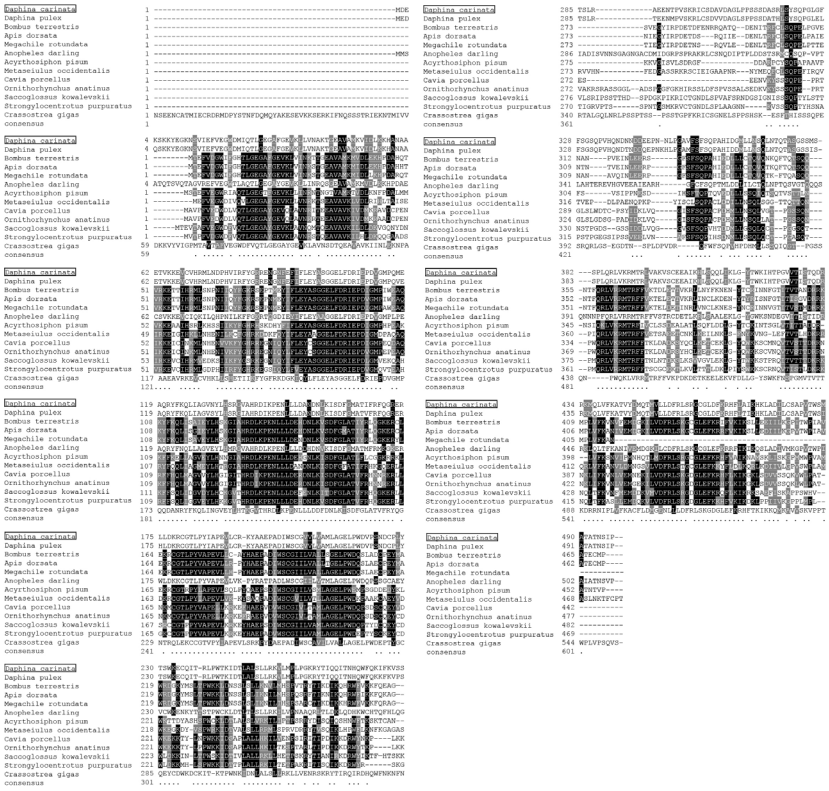

Figure 2. Multiple amino acid sequence alignment of *DcarChk1* to its closest homologs. Identical (black) and similar (gray) amino acids are shown.

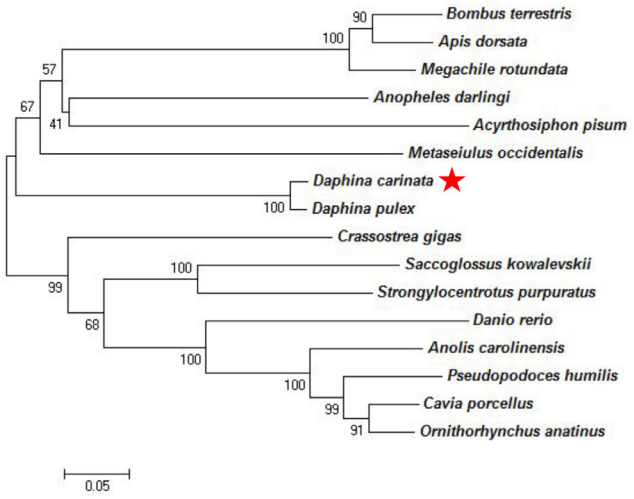

Figure 3. Neighbor-joining tree showing relationship of *DcarChk1* with homologs from other species.

specific mRNA. DcarChk1 mRNA in parthenogenetic females was expressed mainly on the head surface, capillus, and carapace valve edge (Figure 5A and C). In sexual females, expression was concentrated mainly in the joint of the second antennae and on the thoracic limbs and capillus (Figure 5B and D).

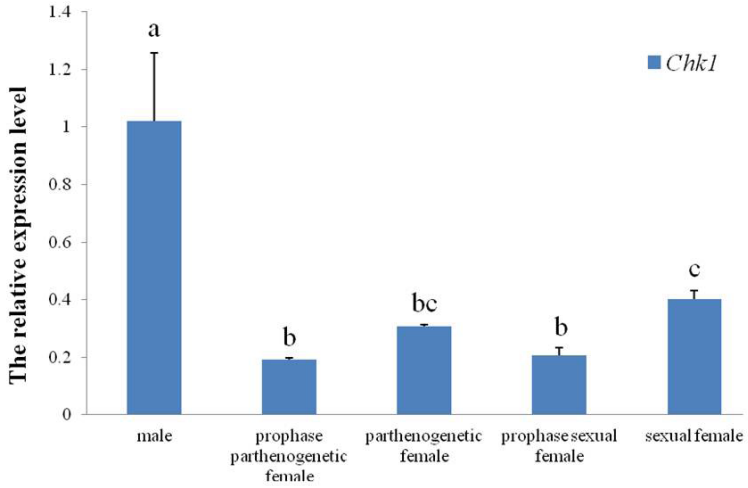

Figure 4. Expression of *DcarChk1* in *Daphnia carinata* males and females at different reproductive phases by real-time qPCR analysis. Labeled bars (a, b, c) indicate significant differences ($P < 0.05$).

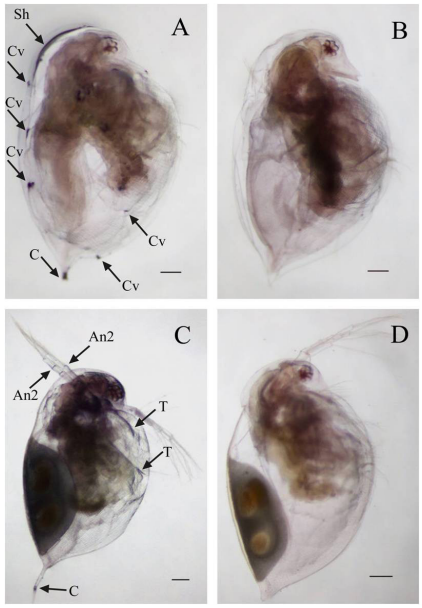

Figure 5. *DcarChk1* in parthenogenetic and sexual *Daphnia carinata* females. Localization by *in situ* hybridization. (**A. C.** DIC-labeled *DcarChk1* antisense probe; **B. D.** DIC-labeled *DcarChk1* sense probe). Blue-violet area shows positive signals. Sh: surface of head; C: capillus; Cv: carapace valve; An2: second antenna; T: thoracic limbs. Scale bars indicate 200 μm.

DISCUSSION

Living organisms possess a complex cell cycle checkpoint signaling pathway, in which DNA damage triggers a series of cascading reactions (Wang et al., 2005). As an effector kinase, the stable expression of *Chk1*, and its protein, aids in repairing damaged DNA and regulating this cycle, thereby ensuring the integrity and stability of the cellular genome (Wang et al., 2006b). *Chk1* encodes a type of serine/threonine-protein kinase belonging to the protein kinase family, within the tyrosine-protein kinases. Protein kinase functionality is evolutionarily conserved across lineages (Manning et al., 2002a) due to the involvement of these proteins in a multitude of cellular processes including division, proliferation, apoptosis, and differentiation (Manning et al., 2002b). Protein kinases catalyze the transfer of gamma phosphate from ATP to one or more aa residues in a protein substrate side-chain, resulting in phosphorylation of substrate proteins. Phosphorylation usually results in a conformational change of the target protein - by changing enzyme activity, cellular location, or associations with other proteins - which affects its function (Hanks et al., 1988). In the current study, we observed that the encoding protein of *DcarChk1* contained a domain with catalytic activity typical of serine/threonine-protein kinases, suggesting that the encoding protein of *DcarChk1* is indeed a kind of serine/threonine-protein kinase; this in turn indicates that *DcarChk1* plays an important role in the growth and development of *D. carinata*.

Protein aa sequence encoded by *DcarChk1* of *D. carinata* had the highest homology with another crustacean species, *D. pulex*, followed by members of the order Insecta. This finding supports the “Pancrustacea” theory, that insects and crustaceans share a common ancestor (Yin, 2003; Song, 2006).

Expression of *DcarChk1* was higher in males than in females; higher in sexual females than parthenogenetic females; and higher in adults than in larvae regardless of the sexual phase of females. The protein encoded by *DcarChk1* is thought to function as a negative regulator of cell division (Pabla et al., 2012). During the early development of *D. carinata* (the larval stage), when cellular division is rapid, lower levels of *DcarChk1* expression were detected than levels observed in adults. This finding suggests that *DcarChk1* plays an important role in the growth and development of this species. The appearance of sexual females and males in this species occurs in response to worsening environmental conditions. Deteriorating habitats can damage DNA, increasing the probability of gene mutation caused by base mismatch. High levels of expression of *DcarChk1* in sexual females and males would ensure timely repair of damaged DNA, and the maintenance of a normal cell cycle. The observed difference in expression levels of *DcarChk1* between males and females, as well as between females in different sexual phases, indicates that expression is sex-dependent and suggests that *DcarChk1* is involved in controlling reproductive transformation of *D. carinata*. Specific underlying mechanisms for this control, however, are yet to be determined.

In situ hybridization has recently emerged as an important means of gene mapping (Gall and Pardue, 1969; Thisse and Thisse, 2008; Zhang et al., 2007; Zhang and Liu, 2013). Using this technique in the current study, we observed that *DcarChk1* was expressed in parthenogenetic females mainly on the head surface, capillus, and carapace valve edge. In sexual females, expression was seen mainly in the joint of the second antennae and on the thoracic limbs and capillus. The carapace valve of cladocerans is similar to that of other crustaceans and is formed from protrusions extending from the rear of the head and the cuticle produced by the outer layer of the epithelium folds. The carapace of *Daphnia,* which is thin, soft and transparent, functions to protect the body and thoracic limbs and can also serve to harbor hatchlings (Jiang and Du, 1979).

During growth, the carapace valve is shed as individuals molt. *Chk1*, as an effector kinase, may act to maintain smooth functioning of the cell cycle during this time. Hence, the high expression of *DcarChk1* in the carapace valve observed in our study may indicate that this gene has an important function in the timely molt of *D. carinata*.

The capillus of *D. carinata* has a role in immune defense and is formed by the extension of the postero-dorsal and -ventral angles of the carapace valve (Zhang et al., 2009). The second antennas, which are stronger than the first antennas, grow on both sides of the head and aid in swimming locomotion (Jiang and Du, 1979). Expression of *DcarChk1* in these areas may contribute to the maintenance of normal physical activity. Unlike other crustaceans, the thoracic limbs of cladocerans are modified and immobile, and function as feeding and respiratory organs (Jiang and Du, 1979). Additionally, the thoracic limbs are used during mating and oogenesis and involved in a dimorphic characteristic between males and females. In males, the endopodite of the first thoracic limb forms a hamulus, and the exopodite develops a long flagellum, which functions to grasp the female during mating (Lin et al., 2008). Gene mapping showing expression of *DcarChk1* in the thoracic limbs of *D. carinata* indicates that this gene has a role in sexual reversal between males and females. Results from the present study lend further support to the importance of *DcarChk1* in the growth and development of *D. carinata*. Further study is required to more robustly understand specific mechanisms of function of *DcarChk1* in this species.

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