MOLECULAR CHARACTERIZATION OF THE GENE ENCODING CHECKPOINT HOMOLOG 1 (*CHK1*) IN *DAPHNIOPSIS TIBETANA* SARS (CRUSTACEA: CLADOCERA) AND ITS EXPRESSION AT DIFFERENT REPRODUCTIVE STAGES

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Abstract. Checkpoint homolog 1 (encoded by *Chk1*) plays an important role in the growth and development of water flea. In this study, the full-length cDNA of *Chk1* encoding an aquaporin from *Daphniopsis tibetana* was isolated by rapid amplification of cDNA ends-PCR (GenBank ID. MW561428.1). The *DtChk1* cDNA was 2025 bp long with a 318-bp 5'- untranslated region (UTR) and a 216-bp 3'-UTR' containing a stop codon (TGA). *DtChk1* cDNA was 1494 bp in open reading frame and encoded a 497-aa polypeptide. At the amino acid sequence level, *DtChk1* showed high homology with homologs in *Daphnia magna* (78%), *Daphnia carinata* (77%), and *Daphnia pulex* (73%). Its homology with *Chk1* sequences from other species ranged from 26% to 45%. Comparison of the deduced amino acid sequence of *DtChk1* with homologs in other species revealed a high degree of conservation of the residues and domains that are essential for biological evolution. Using real-time quantitative PCR, we investigated *DtChk1* gene transcript levels in male and female *D. tibetana* at different reproductive phases. The transcript levels of *DtChk1* were significantly higher in sexual males than in parthenogenetic and sexual females ($P<0.05$), and significantly higher in sexual females than in parthenogenetic females ($P<0.05$). We will discuss the potential uses of *DtChk1* in ecotoxicological studies in the future.
Keywords: *Saline cladoceran, Daphniopsis tibetana, checkpoint homolog 1 (Chk1), reproductive status, ecotoxicology*

Abbreviations: *DtChk1,* gene encoding checkpoint homolog 1 from *Daphniopsis tibetana*; ATM, the ataxia telangiectasia-mutated gene; ATR, ataxia telangiectasia-mutated gene; RT-PCR, reverse transcription polymerase chain reaction; qPCR, quantitative PCR; BLAST, basic local alignment search tool; RACE-PCR, rapid amplification of cDNA ends polymerase chain reaction; ORF Finder, open reading frame finder; pI, isoelectric point; N-J method, Neighbor-Joining method; Q-PCR, quantitative real time polymerase chain reaction; S-TKc, serine/threonine-protein kinase; SQ/TQ, Ser-Gln/Thr-Gln

Introduction

Checkpoint homolog 1, encoded by *Chk1*, is a type of serine/threonine-protein kinase with a highly conserved structure and function. When DNA is damaged, the ataxia telangiectasia-mutated gene (ATM) and ataxia telangiectasia-mutated gene (ATR) are activated immediately and phosphorylates Ser317 (S317) and Ser345 (S345) of *Chkl* to activate the kinase (Furnari et al., 1997; Xiao et al., 2003). After activation, *Chkl* can further act on its downstream protein Cdc25A/B/C, which can end the cell cycle in S and G2/M phase arrest for repairing damaged DNA, thereby regulating the progression of the cell cycle checkpoint (Chen and Sanchez, 2004; Merry et al., 2010). Genes encoding *Chk1*

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proteins are present in the genomes of budding yeast, crustacea, insects, birds, amphibians, and mammals (Chen and Sanchez, 2004; Hajime et al., 2005; Kong et al., 2016). Moreover, other studies about *Chk1* have been used in the treatment of cancer therapies (Chen et al., 2009; Merry et al., 2010).

Cladocera, or water fleas, are an important group of aquatic microcrustaceans in the Crustacea (Xu et al., 2009; Fang et al., 2015; Kong et al., 2016). Previous studies have detected *Chk1* genes in members of the Cladocera, including *Daphnia magna*, *Daphnia pulex*, and *Daphnia carinata* at different reproductive stages. Studies on the function of *Chk1* suggest that it plays a vital role in both the growth and development of these three freshwater fleas, especially in their reproductive plasticity (Hajime et al., 2005; Fang et al., 2015; Kong et al., 2016). *Daphniopsis tibetana*, a saline cladoceran crustacean water flea, inhabits nutrient-poor salt lakes in elevated or alpine regions of Tibet, Xinjiang, and Qinghai Province in China (Zhao et al., 2016; Wang et al., 2019). It is the dominant species of zooplankton in the food web of Chinese salt lake ecosystems (Zhao et al., 2005).

Over the last 20 years, *D. tibetana* has been used as a test organism in marine ecological studies (ecotoxicology), in evolutionary and developmental biology research, and as a food source for marine fish and shrimp larvae (Zhao et al., 2005, 2017; Wei et al., 2018). This species can produce offspring by parthenogenesis or by sexual reproduction. One of the important characteristics of *D. tibetana* is its cyclical reproduction system. It can reproduce by parthenogenesis or by sexual reproduction depending on stimuli from the external environment. While several studies have focused on its cyclical parthenogenesis (Zhao et al., 2017; Wei et al., 2018; Wang et al., 2019), few have focused on the molecular mechanism of its reproductive plasticity.

In this study, we used molecular tools to explore the potential reproductive plasticity of *Chk1* in *D. tibetana* during different reproductive phases. The full-length *Chk1* cDNA from *D. tibetana* was cloned and characterized, and the transcript levels of *Chk1* in sexual individuals (males and females) and parthenogenetic females were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) and compared. The results of this study provide new information about the molecular mechanisms of the reproductive plasticity of *D. tibetana*, and indicate that the *Chk1* gene has potential applications in ecotoxicological research.

Materials and methods

Animal preparation

Daphniopsis tibetana was collected from Lake Namukacuo in September 2018 (Bange County, Tibet, China) and cultured in a closed laboratory at a controlled temperature of 16°C.

Domestication and acclimation of *D. tibetana* were achieved as described by Zhao et al. (2017). First, the salinity level of lake water was decreased by adding boiled pure water. The lake water was slowly replaced with boiled seawater (obtained off shore of Heishijiao, Dalian, China). After 6 months, *D. tibetana* was successfully acclimated and cultured in diluted seawater with a salinity of 20‰. Under these conditions, it was able to reproduce and develop normally.

During domestication and cultivation, *D. tibetana* was fed with four species of algae mixed with a specific volume ratio, 1 (*Chlorella pyrenoidosa*) : 1 (*Chaetoceros muelleri*) : 1 (*Dunaliella salina*) : 2 (*Isochrysis zhanjiangensis*) (Wei et al., 2018).

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The main difference between female and male *D tibetana* is the first pair of antennae. The first antennae of mature males are up to 6 mm long and have long bristles at the end. The long bristles appear as the length of newborn males increases (*Fig. 1A and Fig. 1i*). In contrast, the first antennae of females are undeveloped, very short (<0.5 mm), and have nine olfactory cilia (*Fig. 1Bii and Fig. 1Cii*). The main difference between parthenogenetic and sexual females are that the former produce parthenogenetic eggs (*Fig. 1b*) and the latter produce sexual eggs (*Fig. 1c*).

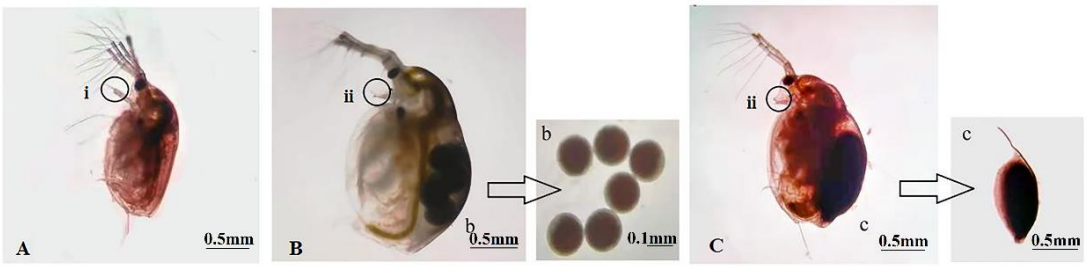

Figure 1. *Morphology of Daphniopsis tibetana. A: male; Ai: first antennae of male; B: parthenogenetic female; Bii: first antennae of female; b: parthenogenetic eggs; C: Sexual male; Cii: First antennae of female; c: resting eggs*

To avoid potential inaccuracies arising from using different individual animals in these experiments, we obtained synchronous female offspring from the same mother. The offspring were continuously cultured and the parents were removed. This allowed us to obtain a population of synchronous pregnant females for subsequent experiments (Wang et al., 2019).

Forty females were selected from the synchronized *D tibetana* population, and each individual was incubated in a separate 100-mL beaker with an adequate amount of mixed algae. After the first larvae were produced, the newborn larvae were carefully removed and examined under a microscope (Germany) to confirm that they were all female. The mother *D. tibetana* that produced all-female larvae were identified as parthenogenetic females.

(1) Parthenogenetic female group: Ten mature parthenogenetic females were aspirated into cell culture plates containing 1 mL culture medium to ensure 10 replicates. They were cultured under the following conditions: culture solution, boiled diluted seawater with salinity of 20‰; temperature, 16°C; photoperiod, 12 h light:12 h dark, with natural light at 700–900 lx.

(2) Sexual female group: Ten mature parthenogenetic females were aspirated into cell culture plates containing 1 mL culture medium to ensure 10 replicates. This group of females was kept inside at 16°C under natural light during the daytime, at then kept overnight at a constant temperature in the dark at 8°C in an incubator (Thermo Scientific, Rockford, IL, USA). The photoperiod was 12-h light and 12-h dark, like that in the parthenogenetic females group.

(3) Sexual male group: Because sexual males and females co-occurred, the sexual male group was treated in the same way as the sexual female group.

To avoid the influence of metabolites on the results, the culture medium was changed every 2 days. After 3 weeks, 10 mature individuals (body length 1.1±0.3 mm) were randomly selected from each of the three groups for molecular analyses.

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Total RNA extraction and first-strand cDNA synthesis

Before extracting the total RNA from parthenogenetic females, the medium was replaced with algae-free medium, and the females were starved for 36 h. This step was necessary to reduce the impact of flea contents on total RNA. Total RNA was extracted using a SteadyPure RNA kit (Accurate Biology, Changsha, China) in accordance with the manufacturer's instructions. The RNA concentration and its degree of integrity were determined by agarose gel electrophoresis and by a nucleic acid protein detector.

First-strand cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Rapid amplification of cDNA ends (RACE)-PCR was carried out using a SMART Ter® RACE 5′/3′ kit (TaKaRa). The 5′-RACE-ready cDNA templates and 3′-RACE-ready cDNA templates were synthesized in accordance with the manufacturer's protocol. The DNA isolated from parthenogenetic females was stored at −20°C.

Primer design

Because there is a high degree of genetic homology between freshwater and saltwater fleas, we chose the *D. magna* sequence (GenBank accession number: XP_032794190.1) to design forward (F1) and reverse (R1) primers to amplify the conserved region of *DtChk1* (*Table 1*). Next, primers were designed for RACE-PCR (5′F, 3′R; *Table 1*) to amplify the whole gene sequence. Based on the full-length sequence and open reading frame (ORF) sequences of *DtChk1*, the gene-specific primers *DtChk1*-F3 and *DtChk1*-R3 (*Table 1*) were designed for quantitative PCR (qPCR). The primers q18SF and q18SR were used to amplify the reference gene (18SrRNA). All primers were synthesized by Sangon Biotech (Shanghai, China). The sequences of primers used for gene isolation, sequencing (Chk1-S and Chk1-V), and determination of transcript levels are shown in *Table 1*. All primers used in this study were designed using Primer 5.0 software.

Table 1. *Sequence and purpose of all primers used in this study*

Primer	Sequence (5'-3')	Purpose	References
F1	CAGAGGGATTGCTCACAGG	Unigene sequence verification	Fang et al., 2015
R1	AGAAGCTAAACATCGCGCCT	Unigene sequence verification	
5'R	GCAACAAGAACGACGCCACAAGACC	5'-RACE reverse primer	
3'F	CATCGCACCTGAGGTTCTCTGTCG	3'-RACE forward primer	
Chk1-S	GGCTGCGATATGATTCAGAC	Sequencing primer	
Chk1-V	GTGGCAATGGACCAAGTAAC	Sequencing primer	
DtChk1-F1	ACAGCGTCAGAACAAATGCG	Gene-specific forward primer	
DtChk1-R1	CGGAAAGTCCTGATGCCTCTA	Gene-specific reverse primer	
q18SF	ACGATGCCAACCAGCAATCCG	Internal primer	
q18SR	TCTGTCAATCCTTCCAGTGTCCGG	Internal primer	

Cloning of cDNA fragment and full-length sequence of DtChk1

Using first-strand cDNA generated from parthenogenetic females as the template, the partial sequence of *DtChk1* was amplified using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) with the following thermal cycling conditions: 42°C for 2 min followed by 4°C, 37°C for 15 min, 85°C for 5 s. The amplified product was stored at

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−20°C. The PCR products were separated by electrophoresis in a 1% agarose gel, then cut out from the gel and purified using a MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China).

The purified PCR products were either directly sequenced or ligated into the pMD™ 19-T vector (TaKaRa), transformed into *Escherichia coli* DH5α competent cells (TaKaRa), and then sequenced (Sangon).

Basic local alignment search tool (BLAST) sequencing results from 3′ and 5′ RACE-PCR were compared at the NCBI database (https://www.ncbi.nlm.nih.gov/), and confirmed the successful cloning of the full-length *DtChk1*. Next, to verify the accuracy of the obtained full-length *DtChk1* sequence, we used the redesigned primers F2 and R2 to reamplify the full-length gene (*Table 1*). There was a high degree of homology between the obtained results and the splicing results, confirming that we had isolated the full-length *DtChk1* cDNA.

Bioinformatics analysis of DtChk1

The NCBI $BLAST_X$ and $BLAST_P$ tools (https://www.ncbi.nlm.nih.gov/) were used for comparative analyses of gene and protein sequences. The NCBI Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to select and predict ORFs and predict amino acid (aa) sequences.

DNAMAN V6 software was used to predict the amino acid composition and isoelectric point (pI) of the putative *DtChk1* protein. The structural domains of the protein encoded by *DtChk1* were analyzed using tools at the SMART website (http://smart.embl-heidelberg.de/). The protein secondary structure analysis and construction of the relevant figures were conducted using tools at the GORIV website (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html). The protein tertiary structure analysis and construction of the relevant figures were conducted using tools at the SWISS-MODEL website (https://swissmodel.expasy.org/). The amino acid sequences of other proteins encoded by *Chk1* genes in other species were obtained from the NCBI ate control was included to assess reagent contamination or primer dimer generation.

To validate the specificity of the products, a melting curve analysis and gel electrophoresis were performed. The *Chk1* gene transcript levels were quantified using the $^{\triangle\triangle}C_T$ method, and were normalized against that of *18SrRNA.*

One-way ANOVA and multiple comparisons (Duncan's test) were conducted using SPSS18.0 software to detect the statistical significance of the differences among groups.

Results and analysis

Cloning of full-length DtChk1

Using the cDNA of parthenogenetic females from *D. tibetana* as the template and gene-specific primers, a 700-bp fragment was obtained by PCR amplification. $BLAST_X$ and $BLAST_P$ searches at the NCBI database revealed that the amplified *Chk1* sequence showed the highest homology with homologs in *D. magna* and *D. pulex*. On the basis of these results, we designed RACE primers. The 5′-RACE and 3′-RACE products were sequenced and used to generate the full-length *DpChk1* cDNA (2025 bp) (GenBank ID. MW561428.1; *Fig. 2*).

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1	AAA	ATA	ATT	TTT	GAC	CCG	ACG	TGC	GTC	CAA	CAC	ATT	TTC	CGA	GCG	45
46	CAC	CGT	AAA	ACG	CGG	CAA	CGA	CGG	CAA	ACA	ACA	GAA	GTT	TGA	CAA	90
91	CAA	GCT	GAC	GAA	ATA	TTT	TGA	GTC	AAA	ATG	ACA	CCT	GTC	TTG	TTA	135
136	ATG	AAA	TCA	ATT	GCG	TGA	TTT	CTG	TAA	TTT	CAG	CTC	AAC	CTG	TGG	180
181	GTG	AAA	ATT	CAT	GAA	AAT	TTA	TCA	ACT	AGC	CAC	AGG	AAA	GAC	GGG	225
226	GAA	GGC	GAT	AAA	TAG	CTG	TCA	TTC	TGT	CGT	TTA	TAC	GGT	CTT	TGT	270
271	CGT	CTC	CGT	GTC	CGT	TCG	GTC	TGC	TGT	GAC	GAT	AAT	ATT	TAT	CCT	315
316	ACC	ATG	GAA	GAA	AAG	AGT	AAA	AAG	TAT	GAA	GGA	AAG	AAC	AGT	GTT	360
0		M	E	E	K	S	K	K	Y	E	G	K	N	S	V	14
361	ATT	GAA	TTT	GTT	GAA	GGT	TGG	GAT	ATG	ATA	CAG	ACC	CTG	GGT	GAA	405
15	I	E	F	V	E	G	W	D	M	I	Q	T	L	G	E	29
406	GGA	GCT	TTT	GGA	GAG	GTG	AAA	CTC	CTA	GTC	AAT	GCA	AAA	ACT	GGA	450
30	G	A	F	G	E	V	K	L	L	V	N	A	K	T	G	44
451	GAA	GCG	GTG	GCA	ATG	AAA	GTA	ATT	GAT	TTA	AAG	AAG	CAT	ATC	AAC	495
45	E	A	V	A	M	K	V	I	D	L	K	K	H	I	N	59
496	GCT	GCC	GAA	ACA	GTA	AAA	AAA	GAA	GTT	TGT	GTT	CAC	CGA	ATG	TTG	540
60	A	A	E	T	V	K	K	E	V	C	V	H	R	M	L	74
541	AAT	GAC	CCT	CAT	GTC	ATT	CGG	TTT	TAT	GGC	AGA	AGG	GAA	AAT	GGC	585
75	N	D	P	H	V	I	R	F	Y	G	R	R	E	N	G	89
586	AAC	TTT	GAA	TTC	ATT	TTT	TTG	GAG	TAT	GCA	AGT	GGT	GGA	GAG	TTG	630
90	N	F	E	F	I	F	L	E	Y	A	S	G	G	E	L	104
631	TTT	GAC	AGA	ATA	GAA	CCT	GAT	GTG	GGA	ATG	CCC	CAA	ATG	GAA	GCT	675
105	F	D	R	I	E	P	D	V	G	M	P	Q	M	E	A	119
676	CAG	CGT	TAT	TTC	AAA	CAA	CTG	ATT	GCT	GGT	GTA	AAC	TAC	TTG	CAT	720
120	Q	R	Y	F	K	Q	L	I	A	G	V	N	Y	L	H	134
721	AGC	AGA	GGA	GTT	GCT	CAT	AGA	GAC	ATA	AAA	CCG	GAA	AAC	TTG	CTT	765
135	S	R	G	V	A	H	R	D	I	K	P	E	N	L	L	149
766	TTG	GAT	GCT	AAT	GAT	AAT	TTA	AAA	ATA	TCC	GAT	TTT	GGA	ATG	GCG	810
150	L	D	A	N	D	N	L	K	I	S	D	F	G	M	A	164
811	ACA	ATC	TTT	AGA	TTT	CAA	GGC	CGA	GAA	AGA	CAT	CTG	GAT	AAA	CGC	855
165	T	I	F	R	F	Q	G	R	E	R	H	L	D	K	R	179
856	TGT	GGA	ACT	TTG	CCT	TAC	ATA	GCT	CCT	GAA	GTG	CTT	TGT	CGC	AAG	900
180	C	G	T	L	P	Y	I	A	P	E	V	L	C	R	K	194
901	TAC	GCA	GCA	GAG	CCG	GCT	GAT	ATT	TGG	TCT	TGC	GGC	GTC	GTC	CTT	945
195	Y	A	A	E	P	A	D	I	W	S	C	G	V	V	L	209
946	GTT	GCC	ATG	TTA	GCT	GGA	GAA	TTA	CCT	TGG	GAC	GTA	CCT	TCC	AAT	990
210	V	A	M	L	A	G	E	L	P	W	D	V	P	S	N	224
991	GAT	TGC	CCC	CTT	TAT	ACA	TCA	TGG	AAA	GAA	TGT	CAG	ATC	ACG	CGA	1035
225	D	C	P	L	Y	T	S	W	K	E	C	Q	I	T	R	239
1036	TTG	CCA	TGG	ACA	AAG	ATT	GAT	ACG	CTT	GCT	TTG	TCT	TTA	CTA	CGG	1080
240	L	P	W	T	K	I	D	T	L	A	L	S	L	L	R	254
1081	AAA	GTC	CTG	ATG	CCT	CTA	CCT	GGG	AAG	CGT	TAT	ACT	ATC	CAA	CAA	1125
255	K	V	L	M	P	L	P	G	K	R	Y	T	I	Q	Q	269
1126	ATA	ACC	AAT	CAT	CAG	TGG	TTT	CAA	AAG	AAA	TTC	AAA	GTT	TCA	AGT	1170

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1171  ACA TCT CTA CGA GCT GAA GAG AAC ACT CCT GTA TCC AAA CGC ATT  1215
285    T   S   L   R   A   E   E   N   T   P   V   S   K   R   I   299
1216  TGT TCT GAC GCT GTG GAC GCT GGT ATA TCA CTG TCT TCG TCA GAC  1260
300    C   S   D   A   V   D   A   G   I   S   L   S   S   S   D   314
1261  CCG AGC CGT CTG TCA TAC TCT CAG CCT GGC TTG GGT TTC TTC TCT  1305
315    P   S   R   L   S   Y   S   Q   P   G   L   G   F   F   S   329
1306  GGT TCC CAA CCG GTT CAT CAA AAC GAC AAC AAT GAT GAA GAG CCG  1350
330    G   S   Q   P   V   H   Q   N   D   N   N   D   E   E   P   344
1351  AAT AAT CAT CTG CCG GGC GCA ATG TTT AGC TTC TCT CAA CCA GCT  1395
345    N   N   H   L   P   G   A   M   F   S   F   S   Q   P   A   359
1396  CAT ATC GAC GAT ATG TTA CTT AAC TCT CAG TTG AAC ACA CAG ACT  1440
360    H   I   D   D   M   L   L   N   S   Q   L   N   T   Q   T   374
1441  GCT TCA GGC TCA AGC ATG AGT TCG CCA CTG CAA AGA CTA GTT AAA  1485
375    A   S   G   S   S   M   S   S   P   L   Q   R   L   V   K   389
1486  AGA ATG ACT CGT TTG GTG GCT AAA GTC AGC TGT GAA GAA GCA ATC  1530
390    R   M   T   R   L   V   A   K   V   S   C   E   E   A   I   404
1531  AAG CAT TTG AGC CAA CAA CTA ATC AAA CTT GGC TAC ACT TGG AAA  1575
405    K   H   L   S   Q   Q   L   I   K   L   G   Y   T   W   K   419
1576  ATA CAC ACC CCA GGA GTG GTT ACT ATA TCA ACC CAG GAT CGA CGC  1620
420    I   H   T   P   G   V   V   T   I   S   T   Q   D   R   R   434
1621  AAA ATG CAA CTT GTT TTC AAG GCA ACA GTT TAT GAT ATG CAG ACA  1665
435    K   M   Q   L   V   F   K   A   T   V   Y   D   M   Q   T   449
1666  ATG GTG TTA CTC GAC TTT CGG CTG TCA AGA GGT TGC GGG CTA GAT  1710
450    M   V   L   L   D   F   R   L   S   R   G   C   G   L   D   464
1711  TTT AAA AGA CAT TTT TTA GCC ATC AAA CAT AAG TTA GCT GAT ATA  1755
465    F   K   R   H   F   L   A   I   K   H   K   L   A   D   I   479
1756  TTG TGC TCA GCT CCA GTA ACG TGG TCC ATA GCC ACT GCT ACT AAC  1800
480    L   C   S   A   P   V   T   W   S   I   A   T   A   T   N   494
1801  AGC ATT CCT TGA ACA ATT CTT TAA ATT ATG TTT GTT TTC TTA GTG  1845
495    S   I   P   *
1846  TTG GAT TAC ACA GAG TGA TTT GTA GCA CAA ATG TTT TAT TTT TTC  1890
1891  CTG GAT GAC AAA GGA CTT ACA GGG GAA TTT GAT CAA AGT ATT AAG  1935
1936  ACT GAA AGC AAG TAA AAC AGA CAT TCA AAA TTC CTG GAG GGC TGG  1980
1981  AAC ACA TTC AGA GAT GAA AAT TAA ATT TTA GGA TTG TTT CAG TTA  2025
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Figure 2. *Full-length nucleotide sequence of DtChk1 and deduced amino acid sequence of DtChk1, protein kinase C phosphorylation site (blue with underline), casein kinaseII phosphorylation site (red with underline), amidation site (black with underline), N-myristoylation site (green with underline), cAMP-and cGMP- dependent protein kinase phosphorylation site (purple with underline), GxGxxG field (yellow background), the SQ/TQ domain had several conserved Ser-Gln (SQ) or Thr-Gln (TQ) motifs (grey background), start codon (ATG with box), stop codon (TGA with box and the asterisk)*

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Analysis of DtChk1 sequence and the encoding protein structure

The full-length cDNA was 2025 bp long, with a 318-bp 5'-untranslated region (UTR) and a 216-bp 3'-UTR containing a stop codon (TGA). The theoretical pI of the encoded protein was 8.66. The 1494 bp ORF encoded a polypeptide of 497-aa (*Fig. 2*).

Structural analyses of *DtChk1* protein sequence revealed a serine/threonine-protein kinase (S-TKc) structural domain (aa positions 21–275, *Fig. 3*). The theoretical secondary structure is shown in *Fig. 4*, and included *α*-helixes consisting of 177 amino acids (34.14%), extended strands consisting of 87 amino acids (17.51%), and random coils consisting of 239 amino acids (48.09%). *β*-Pleated sheets were rare.

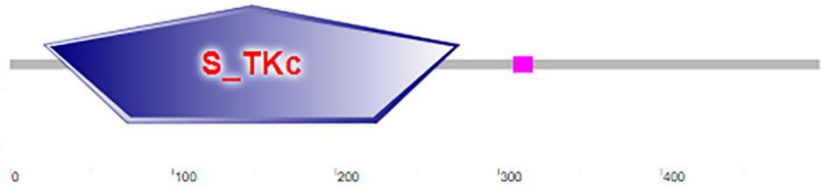

Figure 3. *S_TKs domain of DtChk1 protein*

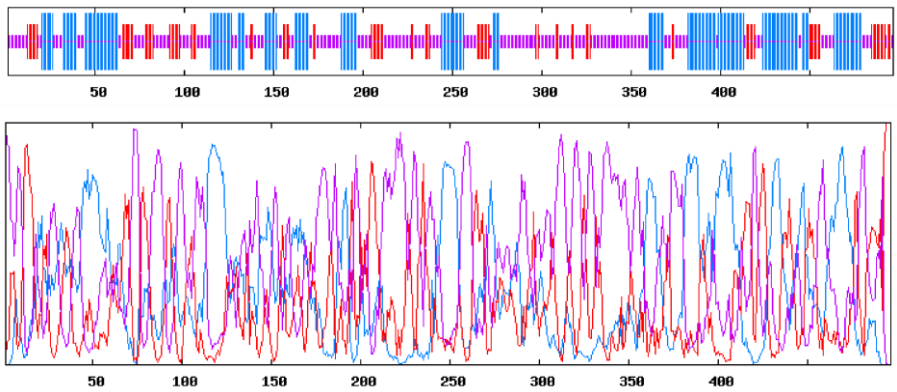

Figure 4. *Secondary structure of DtChk1. Helix regions indicated in blue; coil regions indicated in purple; β-sheets indicated in red*

The tertiary structure analysis revealed that *α*-helixes occupied a larger space than did *β*-pleated sheets, consistent with the predicted secondary structure (*Fig. 5*).

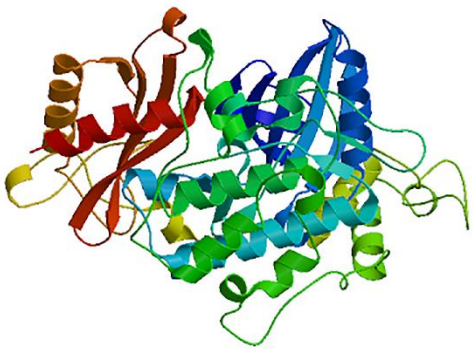

Figure 5. *Tertiary structure of DtChk1 protein*

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Analysis of DtChk1 amino acid homology

The predicted amino acid sequences encoded by *Chk1* genes from eight species, including *D. tibetana*, were compared in a homology analysis (GenBank information is shown in *Table 2*, *Fig. 6*). The predicted *DtChk1* polypeptide had a typical Ser-Gln (SQ)/Thr-Gln (TQ) structural domain, as well as conserved SQ and TQ sequences.

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

Species name	GenBank No.
Daphniopsis tibetana	MW561428.1
Limulus polyphemus	XP_013782750.1
Culex quinquefasciatus	XP_038113736.1
Penaeus monodon	XP_037775034.1
Caenorhabditis elegans	NP_001370816.1
Daphnia carinata	AJP08956.1
Daphnia pulex	AGN95867.1
Daphnia magna	XP_032794190.1
Homo sapiens	NP_001107594.1
Mus musculus	NP_031717.2

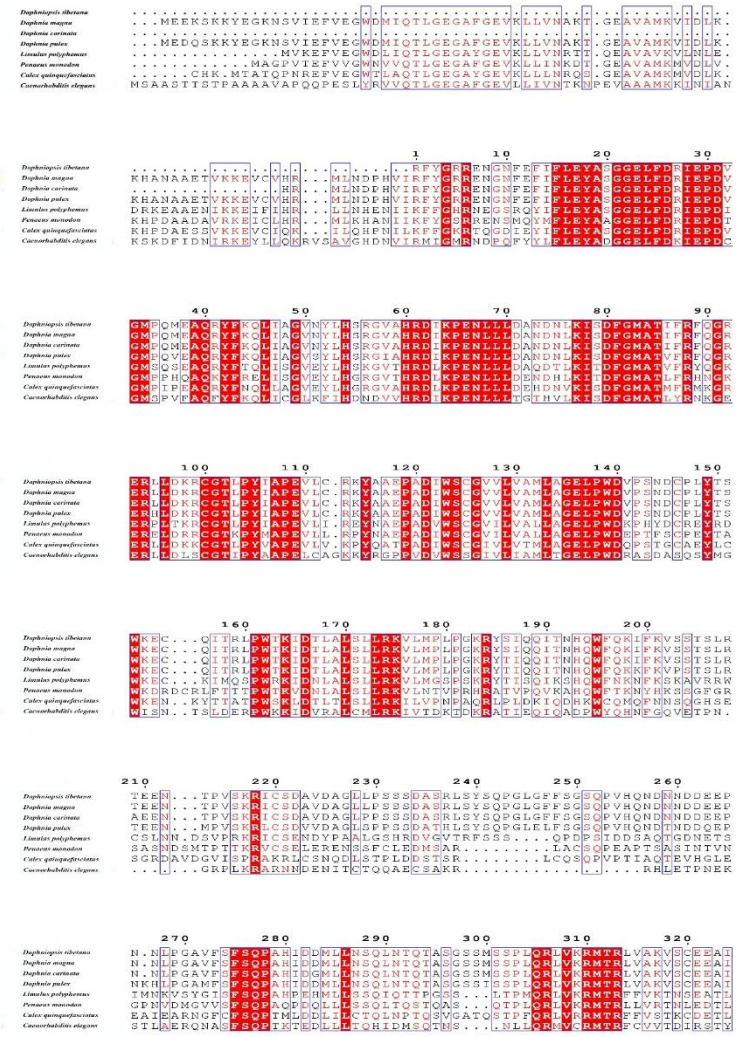

Figure 6. *Comparison of amino acid sequence of DtChk1 with Chk1 proteins in other species*

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DtChk1 showed the highest homology with its homolog in *D. magna* (78%), followed by *D. carinata* (77%) and *D. pulex* (73%), and homology ranging from 26% to 45% to its homologs in the other species (*Limulus polyphemus*, *Penaeus monodon*, *Culex quinquefasciatus*, and *Caenorhabditis elegans*) (*Fig. 6*).

In the phylogenetic tree, *Homo sapiens* and *Mus musculus* were clustered as a single group. Invertebrates were clustered into a single group (*D. tibetana*, *D. magna*, *D. carinata*, and *D. pulex*). The closest relative of *D. tibetana* was *D. magna*. The remaining species (*L. polyphemus*, *P. monodon*, *C. quinquefasciatus*, and *C. elegans*) were also clustered into a single group (*Fig. 7*).

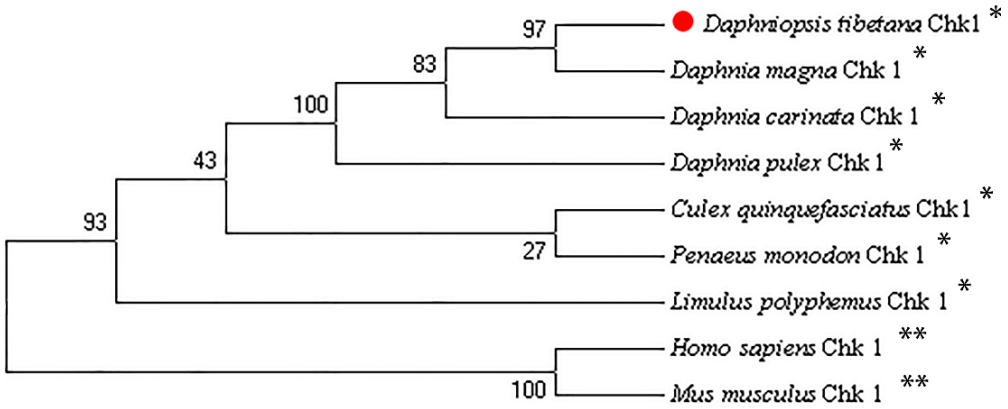

Figure 7. *Neighbor-joining tree showing relationships among DtChk1 and homologs from other species, *indicates invertebrates and * * indicates vertebrates*

Analysis of DtChk1 expression

The transcript levels of *DtChk1* were significantly higher in sexual males than in parthenogenetic and sexual females ($P<0.05$), and significantly higher in sexual females than in parthenogenetic females ($P<0.05$; *Fig. 8*).

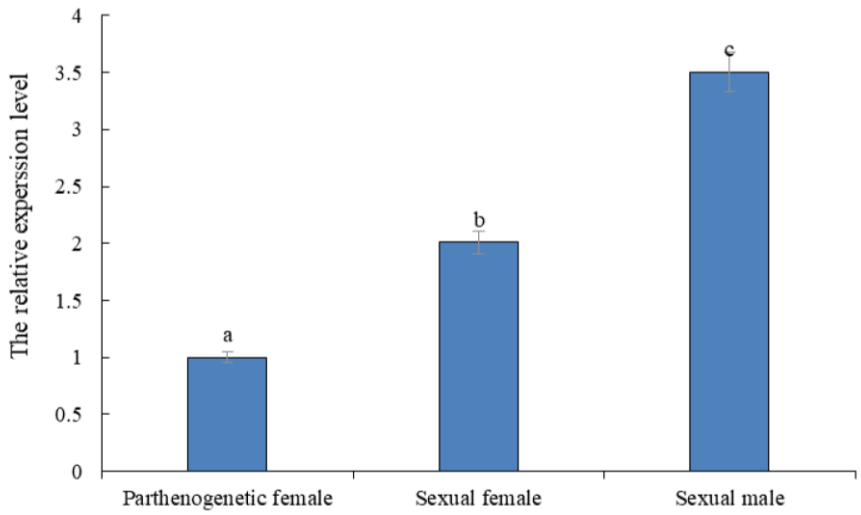

Figure 8. *Transcript levels of DtChk1 in males and females at different reproductive stages*

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Discussion

Daphniopsis tibetana, a salt water flea found in parts of China, is a food source for marine fish and shrimp larvae. Furthermore, it has become a popular test organism for monitoring in marine ecotoxicology research after its domestication. Thus, molecular studies on its reproductive state are of great ecological and technological significance.

In this study, $BLAST_X$ and $BLAST_P$ analyses and multiple sequence comparisons revealed a domain with catalytic activity typical of a serine/threonine-protein kinase in the putative *DtChk1* protein. These results confirmed that *DtChk1* is indeed a serine/threonine-protein kinase.

The amino acid sequence of *DtChk1* was compared with those encoded by homologous genes in related species, and showed the highest homology with those in *D. magna*, *D. carinata,* and *D. pulex*. This finding suggests that *Chk1* plays a vital role in the development and growth of both salt water and fresh water fleas (Hajime et al., 2005; Fang et al., 2015; Kong et al., 2016). As well as showing homology with Chk1 proteins of other crustaceans (four species of water fleas), *DtChk1* also showed homology with related proteins in insects, supporting the theory that crustaceans and insects share a common ancestor, i.e., the "Pancrustacea" theory (Song, 2006).

The transcript levels of *DtChk1* were higher in sexual males than in parthenogenetic and sexual females, and higher in sexual females than in parthenogenetic females. This suggests that sexual males and females have dramatic changes in their genetic information (DNA damage) along with environmental degradation, because *Chk1* and its cooperating proteins are involved in repairing damaged DNA and regulating the cell cycle to ensure the stability and integrity of the cellular genome (Chen and Sanchez, 2004; Merry et al., 2010; Kong et al., 2016).

Our findings are consistent with those reported for *Chk1* genes in *D. magna*, *D. carinata*, and *D. pulex,* indicating that there are certain similarities between salt water fleas and freshwater fleas (Hajime et al., 2005; Fang et al., 2015; Kong et al., 2016). Because *DtChk1* is involved in the process of DNA damage and repair during potential reproduction, its expression level may be a useful indicator of the toxic effects of environmental pollutants such as heavy metals, persistent organic pollutants, and ultraviolet radiation. Further studies are underway to determine the suitability of other potential reproductive genes (*Transformer* gene, *Doublesex1* gene, *Hsp90* gene) (Kong et al., 2015; Kato et al., 2018; Telli et al., 2020) from *D. tibetana* for use in ecotoxicological studies.

Conclusion

We obtained the full-length sequence of the reproduction-related gene *Chk1* of *D. tibetana,* and analyzed the gene sequence, predicted protein sequence and structure, amino acid sequence homology, and the transcript levels of *DtChk1* in male and female *D. tibetana* in different reproductive states. In the future, the results can be used for ecotoxicological studies.

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Author contributions. YIN, D. P: Data curation, Writing-Original draft preparation, Writing-Reviewing and Editing. ZHAO, W: Conceptualization, Methodology, Writing - Reviewing and Editing. ZHANG, Y: Test animals cultivation, Data curation. WEI, J: Visualization, Investigation. WANG, S: Software, Validation. BAO, X. B: Methodology, Software, Supervision.

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Declaration of competing interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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