



# Molecular cloning, characterization and expression analysis of a *Doublesex* gene from *Daphnia carinata* (Crustacea: Cladocera) during different reproductive stages

M.Q. Zhang<sup>1\*</sup>, C.A. Ma<sup>1\*</sup>, W.W. Lv<sup>1</sup>, Y.H. Huang<sup>1</sup>, D.L. Wang<sup>2</sup> and Y.L. Zhao<sup>1</sup>

<sup>1</sup>School of Life Science, East China Normal University, Shanghai, China

<sup>2</sup>School of Marine Sciences, Ningbo University, Ningbo, China

\*These authors contributed equally to this study.

Corresponding author: Y.L. Zhao

E-mail: zhaoyunlong\_ecnu@163.com

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**ABSTRACT.** To better understand the reproductive transformation mechanism of *Daphnia carinata*, a *Doublesex* (*Dsx*) gene was cloned based on rapid amplification of cDNA ends (RACE), and was designated *DapcaDsx2*. Next, we compared similarities and assumed homology based on deduced amino acid sequences. It showed 97.52, 87.94, and 85.11% identity to orthologous genes in *D. magna*, *D. pulex*, and *D. galeata* respectively. Phylogenetic analysis revealed that *DapcaDsx2* clustered in the same class, and was evolutionarily more distant to sequences from other species. qRT-PCR showed that *DapcaDsx2* was most abundantly expressed during sexual reproduction ( $P < 0.05$ ). Using digoxigenin-labeled RNA probes, we studied *DapcaDsx2* expression in parthenogenetic and sexual females by whole-mount *in situ* hybridization. The results revealed that *DapcaDsx2* was mainly

expressed in the second antennae and several sites of the ventral carapace, whereas higher expression levels were found in sexual than in parthenogenetic females. This suggests that the *DapcaDsx2* gene is involved in switching modes of reproduction and in sexual differentiation.

**Key words:** *Daphnia carinata*; *DapcaDsx2* gene; Expression analysis; Whole-mount *in situ* hybridization; Reproductive transformation

## INTRODUCTION

Sex determination is a fundamental biological process, mainly referring to male and female undifferentiated gonads developing into testes or ovaries. In vertebrates, sex determination mechanisms can be divided into genetic sex determination (GSD) and environmental sex determination (ESD) (Marshall-Graves, 2008; Crews and Bull, 2009). Due to their more primitive evolutionary status, sex chromosomes in apparent inferior animals do not differentiate or, in the absence of sex chromosomes, sex determination for both GSD and ESD is more complex (Wallace and Wallace, 2000; Matsuda et al., 2002; Valenzuda et al., 2006; Yoshimoto et al., 2008).

*Daphnia* is considered a model organism for life science research because of its unique reproductive transformation, providing a relatively simple system for the investigation of the relationship between GSD and ESD. *Daphnia* has two special reproductive modes—parthenogenesis and sexual reproduction. If external conditions are suitable, reproduction occurs generally through parthenogenesis. It has a sexual dimorphism. Parthenogenic *Daphnia* produce eggs that develop into offspring without fertilization (Zhang, 2005). However, under adverse environmental conditions, such as temperature changes, starvation, or overpopulation, *Daphnia* produce males and bisexual females, and reproduction switches from an asexual to a sexual mode. In sexual reproduction, the formation of resting eggs, which are fertilized and coated by ephippiums, helps them to survive adverse conditions (Jiang and Du, 1979). Several studies have analyzed cladoceran population growth, reproduction, development, and environmental conditions (Rao et al., 2002; Li and Zhang, 2006). However, most of the studies focused on temperature, food, population density, metabolites, and other external environmental factors (Martinez et al., 1994; Alekseev and Lampert, 2001; Pedrozo and Bohrer, 2003). Previously, Kato et al. (2011) identified two *Doublesex* (*Dsx*) genes (*DapmaDsx1* and *DapmaDsx2*) from a freshwater branchiopod crustacean, *Daphnia magna*. And Toyota et al. (2013) identified homologs of both *Dsx* genes from *Daphnia pulex*, *Daphnia galeata*, and *Ceriodaphnia dubia*.

*Dsx* is a sex-determining gene initially discovered in *Drosophila*, and later researched in *Bombyx mori* (Lalli et al., 2003). *Dsx* is required to produce any sex phenotype, and its mutation can change the sex phenotype, leading to XX embryos developing into adult males, or XY embryos into adult females. In *Drosophila*, the function of *Dsx* results in different phenotypes in males and females, and therefore has opposing regulatory functions (Shearman and Frommer, 1998). According to the different stages of pre-mRNA, *Dsx* is spliced sex-specifically, which affect its expression levels, and is transcribed into sex-specific mRNA isoform (Shimada and Ohbayashi, 2000).

*Daphnia carinata* is an aquatic arthropod (Crustacea: Cladocera: Daphniidae) present in fresh water. In the literature, there are fewer studies about *D. carinata* than *D. magna*, *D. pulex*, and others. Therefore, we aim to identify new details about this species, provide

supplementary research on the *Dsx* gene, and explore the molecular mechanisms of reproductive switching and environmental sex determination in *D. carinata*. In our study, a full-length cDNA sequence of the *Dsx* gene was amplified from *D. carinata* and was named *DapcaDsx2* according to the NCBI database homology analysis. On this basis, a comparative analysis of individuals at different reproductive stages was performed to study the relative and spatial expression of this gene by whole-mount *in situ* hybridization, intending to explore the expression pattern and functions of *DapcaDsx2*. In this study, the role of *DapcaDsx2* in the reproductive transformation of *D. carinata* was investigated, thereby providing a theoretical basis for the reproductive transformation mode and developmental origin of cladocerans and for regulation of animal reproduction development.

## MATERIAL AND METHODS

### Sample preparation and reagents

*D. carinata* were obtained from the Minhang campus channel of the East China Normal University. A healthy laboratory sample of *D. carinata* was incubated at 25°C, under a 14-h light/10-h dark photoperiod and fed cladoceran nutrient solution (1.5 g manure, 2 g vegetables, 20 g fertile soil in 1L boiled, filtered water) for 2-3 weeks. Different sexes of *D. carinata* in response to environmental cues were collected through artificial cultivation. We selected and confirmed differential development of offspring following observations using a Leica DM500 microscope (Leica, Germany). Of these, prophase sexual females and sexual females produce resting eggs, but the ephippia of prophase sexual females is white, while the other is black (Zhang, 2005). Some individuals were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Others were preserved overnight in 4% paraformaldehyde (PFA) at 4°C, followed by the removal of PFA and replacement with 100% methanol, and storage at -20°C for use in whole *in situ* hybridization experiments.

Total RNA was extracted using an RNA extraction kit (Axygen, USA). A Premix Ex *Taq*<sup>TM</sup> Hot Start version kit (TaKaRa, Japan) was used for qRT-PCR. Reverse transcriptase, *Taq* polymerase, and other reagents were purchased from TaKaRa. Rapid amplification of cDNA ends (RACE-PCR) was performed using a SMARTer<sup>TM</sup> RACE cDNA amplification kit (Clontech, USA). The T7 and SP6 RNA polymerases were purchased from Promega, USA. RNA from torula yeast (*Candida utilis*) was purchased from Sigma, USA. DIG RNA labeling mixture, blocking reagent, anti-Digoxigenin-AP Fab fragments, and NBT/BCIP were purchased from Roche, USA. PCR primers were synthesized by Sangon, China, and PCR products were sequenced by Majorbio, China.

### Primer design

The gene sequences of *Dsx* homologs were downloaded from the NCBI database. The sequences of *D. pulex* (GenBank accession No. AB693159.1), *D. magna* (GenBank accession No. AB569298.1), *D. galeata* (GenBank accession No. AB693161.1), and *C. dubia* (GenBank accession No. AB693163.1) were used to design *Dsx*-F and *Dsx*-R primers for a standard PCR to amplify the conserved region with the Primer Premier 5.0 software. A partial sequence of *Dsx* cDNA was amplified. The 3'-RACE and 5'-RACE primers were designed based on the partial sequence, as were the 3'-GSP, 5'-GSP, 3'-NEST, and 5'-NEST primers for nested PCR.

The RT-F and RT-R primers for qRT-PCR were designed in the open reading frame (ORF) of the full-length cDNA sequence. In order to obtain more accurate test results, L8 ribosomal gene sequences were used as the reference gene for fluorescent quantitation, and this was amplified with primers L8-F and L8-R (Weng, 2011). Probe primers, pro-F, and pro-R were designed for the preparation of *in situ* hybridization probes. All primer sequences used in this study are found in Table 1.

**Table 1.** Primer names and sequences.

Primer	Sequence (5'-3')	Purpose
Dsx-F	GCCAGCAACAAGACAGAG	StandardPCR
Dsx-R	GTGGTGGACAAATGACGGT	
L8-F	CGTAGTCGGATGTCTGTCTTCG	L8 gene
L8-R	GCAGATACAACAACGACGCA	
3'GSP	TCGCCATCCGACTTGGGAAGAAAC	RACE-PCR
5'GSP	GCCTAGCAGGAAGCATTCAAGAGCCAT	
3'NEST	TTTCGCTTCGTCTATTTGCCATCCG	NestedPCR
5'NEST	CTTCCCAAGTCGGATGGCGAAATAGA	
RT-F	TCCGACTTGGGAAGAAACTC	qRT-PCR
RT-R	CGGGAGCGTAATGAGATGG	
pro-F	CCATCTCATTACGCTCCCG	Riboprobe
pro-R	GGATTTCGCACTGGCATA	

### Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from individuals stored at -80°C using an RNA extraction kit (Axygen, USA) according to the manufacturer protocol. The first-strand cDNA for standard and qRT-PCR was synthesized using a reverse transcription kit (PrimeScript RT Master Mix Perfect Real-Time Kit, TaKaRa, Japan). The reaction program consisted of 37°C for 15 min and 85°C for 5 s.

### Cloning of the cDNA fragment

Using first-strand cDNA from the adult sexual females as a template, standard PCR was carried out. The reaction volume was 25 µL, and the optimized amplification procedure was as follows: 94°C denaturation for 5 min; 94°C denaturation for 30 s, 55°C annealing for 30 s, 72°C extension for 1 min, 35 cycles; 72°C extension for 10 min; followed by preservation at 4°C. PCR products were separated using 1.2% agarose gel electrophoresis and purified using a SanPrep column DNA gel extraction kit (Sangon, China). They were then sequenced by Majorbio, China. A BLAST search was used to compare the products against the NCBI database, and a partial cDNA sequence of *Dsx* was confirmed.

The 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA templates were prepared according to the SMARTer™ RACE cDNA Amplification Kit User Manual. The RACE-PCR amplification procedure was carried out using a touchdown PCR according to the manual. To increase the amplification efficiency and specificity, the RACE-PCR product was diluted 100-fold as a nested PCR template. The annealing temperature of the nested PCR was 63°C. PCR products were separated and purified as described above. PCR products were inserted into a pGEM-T vector (Promega, USA), and positive clones were identified using PCR, agarose gel electrophoresis, and sequencing.

## Cloning of full-length cDNA and bioinformatics analysis

The 5'-RACE-PCR and 3'-RACE-PCR sequencing results were compared to the NCBI database. The products were spliced with the intermediate sequence to obtain the full-length *Dsx* cDNA, which was named *DapcaDsx2* according to NCBI database homology analysis.

The NCBI BLASTx tool was used to find and compare the homology of nucleic acid and protein sequences. The NCBI ORF finder was used for ORF analysis. SignalP was used for signal peptide identification. The isoelectric point (pI) and molecular weight (MW) of the protein were calculated using the EXPASY database, which was also used to predict the functional domains and active site residues.

The BLAST feature of the NCBI database was used to extract protein names, sequences, and amino acid numbers for subsequent phylogenetic analysis. Using the ClustalW software, we aligned the amino acid sequence encoded by *DapcaDsx2* with 20 other homologous *Dsx* sequences available in GenBank. The sequences used were from *D. magna* (GenBank accession No. BAJ78309.1), *D. pulex* (BAM33608.1), *D. galeata* (BAM33610.1), *C. dubia* (BAM33612.1), *Moina macrocopa* (BAM33613.1), *Paralichthys olivaceus* (ACD62474.1), *Cynoglossus semilaevis* (ACT83604.1), *Tachysurus fulvidraco* (ADM07317.1), *Danio rerio* (AAN61062.1), *Rhinella marina* (ACN54528.1), *Pelodiscus sinensis* (BAD99160.1), *Chelonia mydas* (EMP30456.1), *Mauremys reevesii* (BAG65667.1), *Crocodylus palustris* (ACD74919.1), *Elaphe quadrigata* (BAD99161.1), *Protothrops flavoviridis* (BAF36482.1), *Gallus gallus* (AAF19034.1), *Bos grunniens mutus* (ELR53308.1), *Bos taurus* (ACN86339.1), and *Homo sapiens* (AAR89619.1). A phylogenetic tree was constructed with the MEGA4.0 software using the NJ method with a bootstrap value of 1000.

## qRT-PCR analysis

Primer concentration, template concentration, primer annealing temperatures, and other conditions were optimized. The efficiency of primers for qRT-PCR was determined using the Livak method (Livak and Schmittgen, 2001). The samples were run in biological triplicates. The reaction system contained: 12.5- $\mu$ L SYBR Premix Ex Taq II, 1- $\mu$ L template cDNA, 0.5  $\mu$ L 10  $\mu$ M primer 1, 0.5  $\mu$ L 10  $\mu$ M primer 2, and ddH<sub>2</sub>O to a total volume of 25  $\mu$ L. The following reaction protocol was used: 95°C for 3 min, 95°C for 10 s, 58°C for 30 s, for a total of 40 cycles. Expression was normalized against the ribosomal protein L8 gene (Weng, 2011), and a melting curve was constructed from the results. The experimental data are reported as means and standard deviation, and analyzed with the SPSS software, using one-way ANOVA, with a statistical significance of  $P < 0.05$ . Gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method (Lai et al., 2005).

## Preparation of riboprobes

Using the pro-F and pro-R primers, the PCR amplified a 270-bp DNA fragment. The amplification conditions were: 94°C for 3 min; 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, 35 cycles; 72°C for 10 min. PCR products were detected by agarose gel electrophoresis. Products were purified, ligated into a pGEM-T vector (Promega, USA) using T4 DNA ligase (Promega, USA), and transformed into *Escherichia coli* strain DH5 $\alpha$ , followed by blue-white screening, culturing, plasmid purification, and sequencing. According to the

multiple cloning sites of the pGEM-T vector and digestion sites of *DapcaDsx2*, the restriction enzymes *SphI* and *NcoI* were used to linearize the vector and the gene, respectively. The digested fragments were gel-purified and used as the template for sense and antisense probes, respectively.

Choosing the promoters based on sequencing results, antisense and sense RNA probes were synthesized. The 20- $\mu$ L transcription system contained 4- $\mu$ L 5X transcription buffer, 2  $\mu$ L 100 mM DTT, 0.5  $\mu$ L 40 U RNase inhibitor, 8  $\mu$ L linear DNA (<1  $\mu$ g), 2  $\mu$ L 10X DIG labeling mixture, 1  $\mu$ L 20 U/ $\mu$ L SP6 or T7RNA Polymerase and 2.5  $\mu$ L RNase-free H<sub>2</sub>O. Reactions were mixed and incubated at 37°C for 2 h. One-microliter DNase (RNase-free) was added to the system, and incubation was continued for a further 15 min. After adding 1/9 volume of 5 M LiCl and two volumes of absolute ethanol, the incubation was continued at -20°C overnight. Mixtures were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was discarded. This was followed by washing with 75% ethanol, 60  $\mu$ L 75% ethanol was added, and the samples were centrifuged at 13,000 rpm for 5 min. After thorough drying to remove residual ethanol, the RNA sediment was resuspended in 30  $\mu$ L DEPC water with 1.5  $\mu$ L 20 U RNA inhibitor added, and stored at -80°C.

### Whole-mount *in situ* hybridization

The whole-mount *in situ* hybridization technique employed the methods used by Thisse et al. (Chavez et al., 1999; Mitchell and Crews, 2002; Thisse and Thisse, 2007), with changes based on the characteristics of *D. carinata*. Individual specimens were digested with proteinase K for about 15 min. Through the exploration of gradient temperature, an optimum temperature of 65°C was established. Pre-hybridization at 65°C for 2.5 h was followed by the addition of 100  $\mu$ L of a 1:200 dilution of RNA probe in pre-hybridization solution, and incubation was continued at 65°C overnight. An antisense probe was used in the experimental group and a sense probe in the control group, to ensure specific hybridization signals. The following day, the samples were aspirated and gradient-washed. They were then blocked for about 2 h at room temperature using MAB blocking solution. Hybridization was detected using a 1:2,000 dilution of anti-DIG antibody (Roche, USA) at 4°C for 13 h, followed by washing with MABT, TSM1, and TSM2, respectively. Samples were stained with NBT liquid dye (Roche, USA) at room temperature in the dark for 10 min, and stored in 4% PFA. Hybridization was visualized and recorded using a BX51 fluorescence microscope (Olympus, USA).

## RESULTS

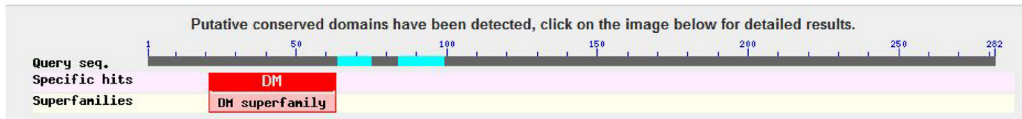
### Cloning full-length cDNA of the *DapcaDsx2* gene

Using cDNA from a sexual female as the template, a 444-bp fragment was acquired by PCR amplification using specific primers. BLASTx analysis revealed high similarity with the published *Dsx2* gene sequences of *D. pulex*, *D. magna*, and *D. galeata*. On this basis, 3'-RACE and 5'-RACE primers were designed for the amplification of fragments of 710 and 797 bp, respectively. Via sequence contrasting and splicing, we obtained a 1108-bp full-length cDNA of *DapcaDsx2* (GenBank accession No. KF753236).

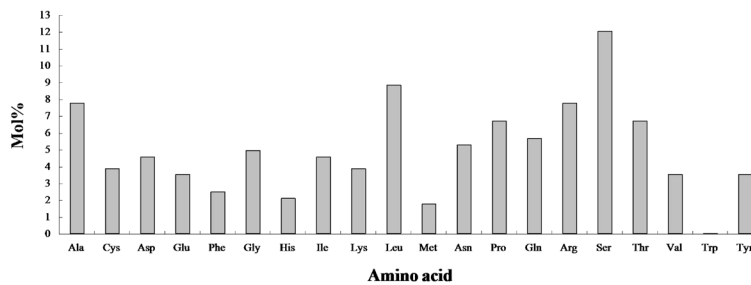
## Characteristics of the *DapcaDsx2* sequence

The full-length cDNA comprised a 259-bp 3'-untranslated region (UTR), a 3'-polyA tail, but no 5'-UTR. It also contained an 849-bp ORF and four N-glycosylation sites. Residues 20-73 contained a highly conserved DM domain, and residues 196-234 contained an oligomerization domain, both of which could improve the specificity of DNA binding. The ORF encoded a protein composed of 282 amino acids with a calculated MW of 31,069.06 Da, and a theoretical pI of 9.14. Analysis of the amino acid composition encoded by *DapcaDsx2* using the DNAMAN software showed that there were 33 alkali amino acids including 11 lysine (Lys), 22 arginine (Arg), and 23 acidic amino acids including 13 aspartic acid (Asp) and 10 glutamate (Glu). Regarding amino acid content, the proportion of hydrophobic amino acids (27.3%) was lower than that of hydrophilic amino acids (37.2%). (Figure 1)

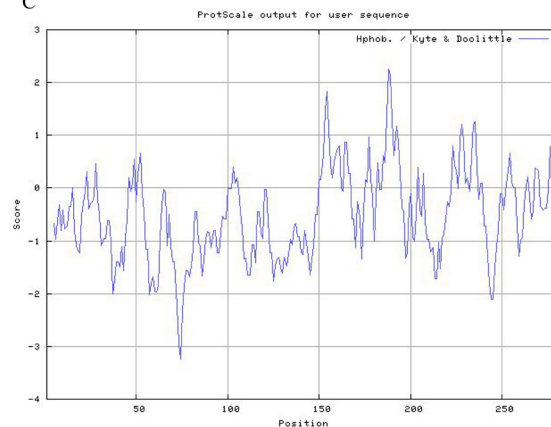
A



B



C

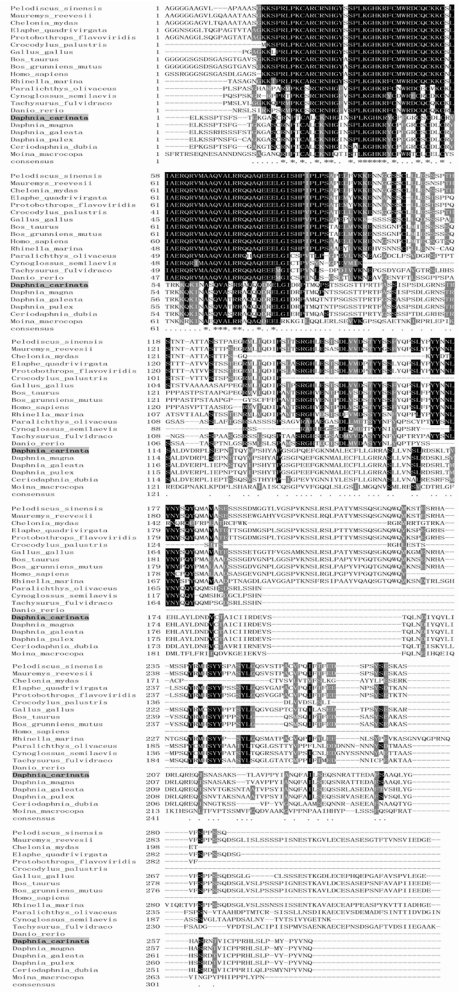


**Figure 1.** A. Distribution of DM domain. B. Composition of amino acid of *DapcaDsx2*. C. The graph indicated the hydrophilicity and hydrophobicity of the amino acid sequence (The abscissa axis represented the position of the amino acid sequence; the ordinate axis represented the value of hydrophilicity and hydrophobicity. The positive value indicated hydrophobicity, and the negative value indicated hydrophilicity).

**Similarity of the *DapcaDsx2* protein sequence with other *Dsx* ortholog proteins**

The results showed that the highest homology was shared with *D. magna* (97.52%), followed by *D. pulex* (87.94%), *D. galeata* (85.11%), and *C. dubia* (69.50%). Homology with the other 16 species ranged from 15 to 28% (Figure 2).

A phylogenetic tree of 21 *Dsx* amino acid sequences was reconstructed using the NJ method with the MEGA 4.0 software. The results showed that the *D. carinata Dsx2* gene was most closely related to the same gene in *D. magna*, forming one cluster. Both of these had a relatively close genetic relationship with the cladocerans *D. pulex*, *D. galeata*, *C. dubia*, and *M. macrocopa*, and formed one larger cluster. In terms of molecular evolutionary distance, the *DapcaDsx2* cDNA was closely related to crustacean sequences, and more distantly related to insects or other sequences (Figure 3).



**Figure 2.** Multiple sequence alignment of *DapcaDsx2* amino acid sequence with its closest homologs. Identical amino acids are highlighted in black, similar amino acids shown in gray.



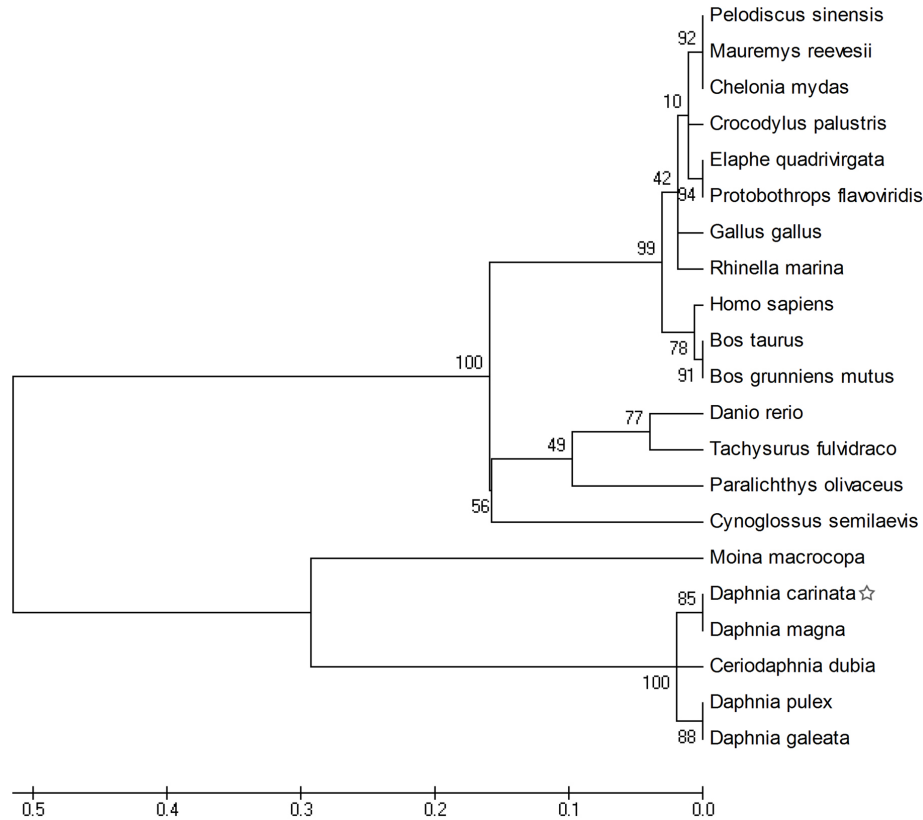
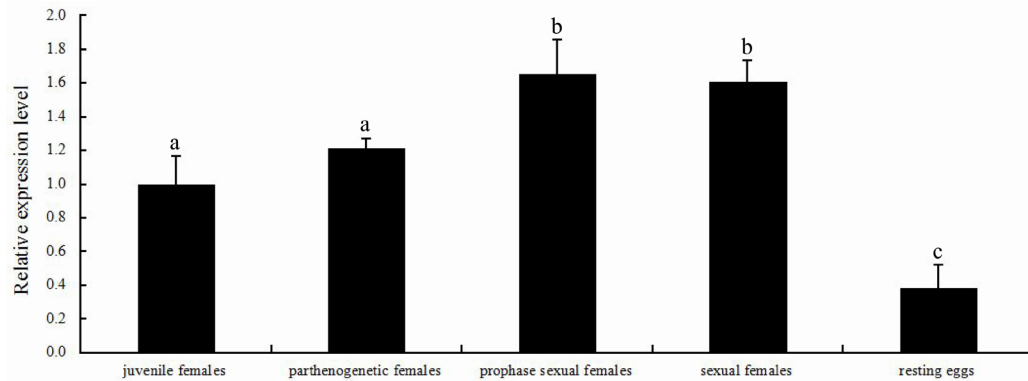


Figure 3. Neighbor-joining phylogenetic tree of *DapcaDsx2* and *Dsx* sequences from other species.

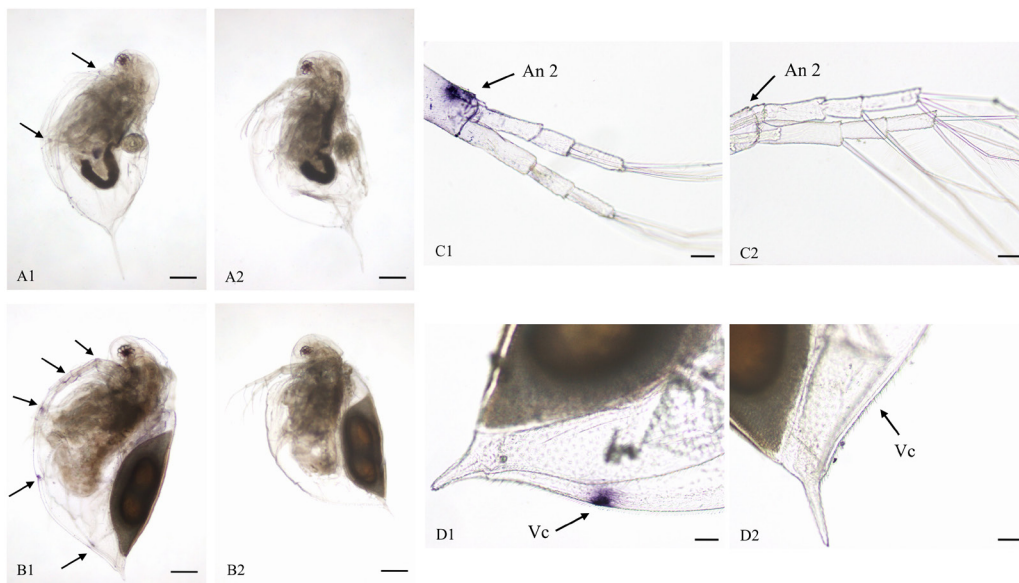
### Analysis of *DapcaDsx2* expression

Using qRT-PCR, we analyzed the expression of *DapcaDsx2* during five reproductive stages: juvenile females, parthenogenetic females (with summer eggs), prophase sexual females, sexual females, and resting eggs (Figure 4). *DapcaDsx2* was most abundantly expressed in prophase sexual females and sexual females, followed by juvenile females and parthenogenetic females. The lowest expression was detected in the resting eggs. Expression levels of *DapcaDsx2* in asexual and sexual reproduction were significantly different ( $P < 0.05$ ). Taken together, the results clearly show that *DapcaDsx2* mRNA is expressed during the different reproductive stages.

*DapcaDsx2* expression patterns were studied by whole-mount *in situ* hybridization using digoxin-labeled RNA probes. *DapcaDsx2* was mainly expressed in the second antennae and several sites of the ventral carapace. In contrast, the expression level in sexual females was higher than that in parthenogenetic females. In addition, the sense probe detected no signal at the corresponding sites. These findings are consistent with the quantitative expression results, which indicated specific expression profiles of *DapcaDsx2* mRNA in different reproductive stages (Figure 5).



**Figure 4.** qRT-PCR of *DapcaDsx2* in individual organisms during five reproductive stages. Labeled bars (a, b, c) indicate significant differences ( $P < 0.05$ ).



**Figure 5.** Whole-mount *in situ* hybridization in the body of *Daphnia carinata* (A1-D1: DIG-labeled *DapcaDsx2* antisense probe; A2-D2: DIG-labeled *DapcaDsx2* sense probe). The blue-violet area shows positive signals. **A1.** **A2.** Parthenogenetic *Daphnia*; **B1.** **B2.** sexual female *Daphnia*. Bar = 200  $\mu\text{m}$ . **C1.** **C2.** **D1.** **D2.** Parts of the structure in sexual female *Daphnia*. An 2: second antenna; Vc: ventral carapace. Bar = 50  $\mu\text{m}$ .

## DISCUSSION

The *Dsx* gene is important and is known to be a sex-determining gene in insects. In the three-cascade gene pathway of sex-determination, it directly determines the sexual development of *Drosophila*. In females, the gene products of *Dsx* and *Intersex* synergistically promote the expression of female differentiation genes, resulting in female differentiation. In males,

*Sex-lethal* (*Sxl*) is not activated, so that *Transformer* (*Tra*) is activated, resulting in a male-specific expression of *Dsx*, which leads to male differentiation (Sieved et al., 1997). Research has shown that expression of the *Drosophila* gene *Sxl* varies according to sex, a dimer being formed as a result of the female-specific regulation of *Sxl* generated by the TRA and TRA-2 binding protein encoded by *Tra-2* (Lalli et al., 2003). The *Dsx* gene is also an important site of action of TRA and TRA-2 and is evolutionarily conserved, with homologs in *Bactrocera tryoni*, the Phoridae, and *Bombyx mori* Linnaeus (Sieved et al., 1997; Shearman and Frommer, 1998; Ohbayashi et al., 2001).

By comparing the sequences, the *DapcaDsx2* amino acid sequence of *D. carinata* were found to have high similarity with those of *D. magna*, *D. pulex*, and *D. galeata*, but relatively low homology with species from other classes. The topology of the phylogenetic relationship for *Dsx* orthologs was in agreement with the taxonomic relationships. A phylogenetic analysis based on amino acid sequences revealed that *DapcaDsx2* and other known *Dsx* proteins are conserved, especially among the cladocera. In the phylogenetic tree, *D. carinata* clustered with *D. magna*, *D. pulex*, *D. galeata*, *C. dubia*, and *M. macrocopa* within the same class. In a homology comparison with other species, *Dsx* amino acid sequences from different species possess a highly conserved region called the DM (*Dsx* and *Mab-3* related domain), which is derived from the *Drosophila melanogaster* *Dsx* gene and the *Caenorhabditis elegans* *Mab-3* gene. This is an evolutionary highly conserved DNA binding domain, which can be found from vertebrates to invertebrates (Ottolenghi et al., 2002; Kazuyuki et al., 2002). The DM domain contains a pair of cysteine residues in the N-terminal, and a pair of histidine residues in the C-terminal, which form a zinc finger structure (Raymond et al., 1998). The minimal DNA binding element is important in the *Dsx* protein, which is considered to determine gender (Raymond et al., 1999a,b).

qRT-PCR analysis of *DapcaDsx2* gene expression revealed that the highest expression was found during sexual reproduction, followed by asexual reproduction. *Dsx* function has been co-opted to facilitate the evolution of environmentally cued intrasexual dimorphisms (Kijimoto et al., 2012), and it is known that *Daphnia* reproduction switches from parthenogenic to sexual females in response to environmental cues, so *DapcaDsx2* expression levels differ between asexual and sexual reproduction (Liu et al., 2014). Taken together, these results clearly show that *DapcaDsx2* mRNA has a specific expression pattern during the different reproductive stages, and that *DapcaDsx2* mRNA levels are significantly higher during sexual than asexual reproduction, indicating that it exhibits gender-specific expression, which would play an important role in regulating reproductive transformation in *D. carinata*.

*In situ* hybridization as a means to detect the RNA expression in cells and tissues has been widely described for *D. rerio*, *Artemia*, *Drosophila* and other species (Chavez et al., 1999; Mitchell and Crews, 2002; Thisse and Thisse, 2007), but studies on small cladocerans are much less common. In this study, we analyzed the spatial expression sites of *DapcaDsx2* to ensure the integrity of *D. carinata* individuals by using digoxigenin-labeled RNA probes for the first time. Because the *D. carinata* body is encased in two slices of chitinous carapace, which might limit the efficiency of the RNA probe binding, proteinase K was used to permeabilize cells before hybridization. However, the concentration and treatment time of proteinase K are key to the success of the experiment. We found that the concentration of 10- $\mu$ g/mL proteinase K was optimal, and that sexual organisms and parthenogenetic organisms were digested for 15 and 10 min, respectively. Moreover, if the hybridization temperature is too low, nonspecific hybridization and background staining may take place, and if it is too high, incom-

plete or no hybridization may result. Therefore, a temperature gradient was performed, and an optimum temperature of 65°C was established. To verify the specificity of the experiment, a parallel control was made. It showed no specific expression in sexual and parthenogenetic females by sense probes.

Following optimization, the *in situ* hybridization experiments showed that *DapcaDsx2* was mainly expressed in the second antenna and parts of the ventral carapace, and higher expression levels were found in sexual than in parthenogenetic females. *DapcaDsx2* mRNA was specifically expressed in different reproductive stages and might therefore play an important role in switching between the two reproduction strategies. The expression of *DapcaDsx2* was visible in the second antenna and carapace. The second antenna of *D. carinata* is a moving and sensory organ (Jiang and Du, 1979). The carapace is equivalent to the carapace of other crustaceans, which cannot only be used to protect thoracic limbs, which lose the ability to move and become the tool of food intake, but can also be used to incubate their young (Shi, 2000). Some studies on the *Dsx* gene suggest that *Dsx* expression affects organ formation (Fujii and Shimada, 2007; Kato et al., 2011). Therefore, visible expression in such important organs could suggest a regulatory role of *DapcaDsx2* in the growth and development of *D. carinata*. However, the detailed molecular mechanisms and reasons for the expression of this gene in these organs need further study using RNA interference, ectopic expression, and immunohistochemical techniques.

## CONCLUSIONS

This gene cloning and expression study showed that, *DapcaDsx2* is expressed in higher concentrations in sexually reproducing animals, and that it might be responsible for some sexual dimorphism. It is also likely that *DapcaDsx2* was switched activated because of other regulatory genes. This will be the subject of further research. In general, environmental sex determination is widespread in many fish and invertebrate species but is poorly understood. This study represents a step towards elucidating the molecular mechanisms involved.

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