Identification and Functional Analysis of the Doublesex Gene in the Redclaw Crayfish, Cherax quadricarinatus

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Abstract

Background: Crustaceans often exhibit significant sexual dimorphism during their growth process. However, their sex determination system is relatively complex, and still lacks of related studies that involved in sex determination and differentiation.

Results: In the present study, the gene of *Doublesex (Cqdsx)* was identified and characterized for the first time in the redclaw crayfish, *Cherax quadricarinatus*. The full-length cDNA was 1271 bp, comprising a 155 bp 5'-untranslated region (5'-UTR), a 885 bp predicted open reading frame (ORF) encoding 294 amino acid polypeptides, and a 231 bp 3'-UTR. The deduced amino acid sequence of Cqdsx was predicted to contain a highly conserved DM domain and shared nearly 50% identity to DM-peptides from other species. The results of quantitative Real-time PCR in various tissues revealed that *Cqdsx* was strongly expressed in gonads, while was almost undetectable in gill, heart, hepatopancreas, muscle and intestine. Comparing expression level in different embryonic stages found that *Cqdsx* was gradually increased with the development of the embryos. In situ hybridization to gonad sections showed that intensive hybridization signals were mainly observed in oocytes and ovarian lamellae and weak signals were detected in spermatocyte. Additional, *Cqdsx* gene exhibited the higher transcript levels in the early stage of ovarian development. Furthermore, RNAi-targeting *Cqdsx* silencing induced a decrease of *Cq-IAG* trascripts, which regulated the male sexual differentiation in crustacean.

Conclusion: DM-domain genes play an important role in the sex determination and differentiation among animal kingdom. The full-length cDNA of *Cqdsx* in *C. quadricarinatus* was isolated and characterized. Our findings strongly suggests an essential role for *Cqdsx* in the female ovarian development/differentiation of the redclaw crayfish. These data may provide us a better understanding of sex determination in *C. quadricarinatus*.

Background

Crustaceans often exhibit significant sexual dimorphism during their growth process [1]. Achieving large-scale monosexual aquaculture of crustaceans by sex-control technique can not only increase productivity and yields, but also reduce the ecological risk of alien species invasion [2,3]. Therefore, the study of the sex determination mechanism on crustacean species has already attracted much attention of many researchers. However, due to crustaceans are located in special position during the process of phylogenetic evolution, their sex determination system is relatively complex compared with vertebrates, and at present still lacks of related studies that involved in sex determination and differentiation of crustaceans. Previous studies have shown that the molecular mechanisms involved in sex determination vary widely among different species. For example, a cell autonomous and alternative splicing mechanism is responsible for sexual dimorphism in insects, while sex determination and maintenance of mammals are more dependent on endocrine regulation of gonads [4]. Although there are different upstream signal pathways regulating sex determination, Raynond et al. have shown that some sex-related genes located at downstream are highly conserved [5].

The DM domain is an ancient, conservative component in evolution, and homologous genes containing DM-domain mainly include the *dmrt* gene in vertebrate [6], the Doublesex (*dsx*) gene in *Drosophila* [7], and the *mab-3* gene in nematodes [8], in which the *dsx* gene is the first key regulatory factor found in the most downstream of the *Drosophila* sex determination cascade. Previous studies on the *dsx* gene mostly focus on *Drosophila* or its related species as well as *Bombyx mori* of *Lepidoptera* [9,10]. These results indicate that the protein encoded by this gene contains two multimerized domains: one is the DM domain shared by *dmrt* gene family, and the other is a unique sex-specific splicing OD2 domain. Both Dsx^M and Dsx^F regulate sex-specific morphology by inhibiting or activating the expression of target genes [11].

To date, some progress has been made in sex-related researches of crustacean species. For example, three different mature forms of *dsx* gene are cloned in water flea (*Daphnia magna*), in which *dsx1* gene showed sexual dimorphism in the abundance of transcripts during embryogenesis. Subsequently, knockout of the *dsx1* gene in the male-embryo leads to ovarian development, indicating its role in regulation sex differentiation in males, which pointed us the direction to explore the sex determination mechanism of crustaceans [12]. In *Fenneropenaeus chinensis*, the *dsx* genes mainly express in the testis, and the mRNA level is gradually increased with the larval development. The results of RNAi silencing show that it can regulate the expression of *FcIAG*, a gene important for sexual differentiation of male crustacean [13]. In recent years, high-throughput sequencing techniques have been widely used in the study of decapod species, and many sex-related homologous genes have been identified, including *sxl*, *tra*2, *foxl*2, *fru*, etc. It is worth noting, however, that the sex-linked difference of these genes in crustaceans lies in the abundance of their transcripts rather than in alternative splicing forms [14]. Therefore, there is a lack of direct evidence to reveal the molecular mechanism of the sex determination of crustaceans, and further research is needed to elucidate the mechanism of these genes.

The redclaw crayfish (*Cherax quadricarinatus*), commonly known as Australian freshwater lobster, is native to the tropical regions of northern Australia and southern New Guinea [15]. As a member of the world's valuable freshwater shrimps, *C. quadricarinatus* has the characteristics of fast growth, strong resistance to stress, tender meat and high meat yield. As other freshwater decapods, the redclaw crayfish males grow faster and bigger than females. However, its mechanism of phenotypic differentiation and dimorphic development is still unclear. In this study, the full-length cDNA of *Cqdsx* gene from *C. quadricarinatus* was cloned, and its expression patterns in various tissues, different embryonic and ovarian development stage, as well as its localization in gonad tissues, were analyzed. Besides, the effect of *Cqdsx* gene silencing via RNA interference (RNAi) on the expression of *Cq-IAG* was investigated in *C. quadricarinatus*. These results will provide theoretical basis for further study on sex determination mechanism of *C. quadricarinatus*.

Materials And Methods

Samples Collection and Nucleic acid Preparation

The experimental specimens of adult *C. quadricarinatus* (body weight, 55±10 g; body length 12.5±2 cm) were obtained from the Balidian breeding base of Zhejiang Institute of Freshwater Fisheries (Huzhou, Zhejiang Province). Various tissues (testes, ovaries, heart, muscle, gill, intestine and hepatopancreas) were dissected on an ice bath, immediately frozen in liquid nitrogen, and stored at -80℃ until used for RNA extraction. Embryo samples at different developmental stages from the offsprings of the overwintered gravid crayfish (fertilized eggs, cleavage and blastula, gastrula, nauplius, eye pigments forming, and prehatching) were collected and preserved in liquid nitrogen for RNA extraction. Description of *C. quadricarinatus* embryonic development was based on a previously published method [16]. Ovaries at different periods (Stage I to VI) were sampled based on the biological process and histological characteristics.

Total RNA was extracted from tissues and embryos of *C. quadricarinatus* using Trizol reagent (Total RNA Extractor Kit, Sangon Biotech), and the RNA quality was assessed by electrophoresis on 1% agarose gel. Total RNA isolated from all samples was reverse transcribed using HiFiScript cDNA first-strand synthesis kit (Cwbio, Beijing) according to the manufacturer's procedures for next reverse transcription-PCR (RT-PCR) or Real-time quantitative PCR (qRT-PCR), respectively. Animal experiments were approved by the Committee of Laboratory Animal Experimentation at Zhejiang Institute of Freshwater Fisheries, Huzhou, China.

1.1 Full-length dsx cDNA Sequence Amplification

Firstly, downloaded the *dsx* coding sequence from known species, such as *Drosophila melanogaster* (Genbank accession No. NM_169202), *Bombyx mori* (Genbank accession No. NM_001043406) and *Fenneropenaeus chinensis* (Genbank accession No. JQ965255), and searched of homulogous fragment sequence based on RNA-seq data from *C. quadricarinatus* (data not published). As a result, an EST sequence containing a DM domain was found. On this basis, gene-specific primers (Table 1) were designed to amplify the full-length cDNA sequence using the SMARTer^{TM} RACE Amplification Kit (Clontech, USA) following the manufacturer’s protocol. The amplified PCR products of 3’/5’ RACE reactions were subjected to clone into the pMD18-T vector for sequencing, respectively. Finally, the full-length *dsx* cDNA was assembled from the sequenced results of RACEs by DNAMAN 5.0 software.

Bioinformatic Analysis

The nucleotide sequence and deduced amino acid sequence of *dsx* were analyzed using Jellyfish software (3.3.1). The secondary structure and basic physicochemical parameters of Dsx protein were predicted using the online software InterProScan (http://www.ebi.ac.uk/interpro/scan.html) and ExPASy (https://web.expasy.org/compute_ pi/), respectively. The conserved amino acid sequence of Dsx DM-domain was aligned with that of DM-domain from other species using DNAMAN, and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.0 software. The reliability of the branching was tested using bootstrap re-sampling (with 500 pseudo replicates).

Quantitative RT-PCR

The cDNA from different tissues, embryos and ovary developmental stages synthesized by reverse transcription were used as template, and qRT-PCR was performed for detection transcript levels of *dsx* gene using SYBR Premix Ex Taq kit (TaKaRa, Japan). The primers used for qRT-PCR was shown in Table 1, and *18S-rRNA* (Genbank accession No. AF235966) was used as a normalizing gene. SYBR Green based qRT-PCR was performed using LightCycler® 480 System (Roche, Switzerland), and set the program of 95°C for 10 s followed 40 cycles of 95°C for 5 s and 60°C for 20 s. Each test and their endogenous control were performed in triplicate. Gene expression data was analyzed using the $2^{-\Delta\Delta CT}$ method [17] and the histogram using GraphPad Prism 5 software was obtained.

Tissues Section Preparation and In situ Hybridization

Testes and ovaries from adult *C. quadricarinatus* were carefully dissected and fixed in 4% paraformaldehyde overnight at 4℃, and then embedded in paraffin for cross-sections. A target fragment was amplified with probe primers (Table 1) and cloned into pbluescriptSKII plasmid. Dig-labeled probe was transcribed in vitro and synthesized according to the instructions of the Roche DIG RNA Labeling Mix. For in situ hybridization, the general procedures consisted of 4% paraformaldehyde fixation, proteinase K digestion, room temperature prehybridization, and followed by hybridization with Dig-labeled probe at 55℃ for 12-16 h, and the antibody was eluted after blocking solution treatment. Finally, the hybridization signals were visualized by microscopy and recorded with chromogenic solution NBT/BCIP.

dsRNA Preparation and In-vivo Injection

Gene specific primers with T7 promoter sequence (Table 1) were designed to amplify a 400 bp cDNA fragment of *dsx* gene followed the PCR program described below: 94℃ for 4 min; 35 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s; followed by one cycle of 72℃ for 10 min. PCR product was purified and cloned into pUC57 vector, and the EGFP DNA fragment with T7 promoter sites was synthesized by the GenScript Company. Linearized vectors with E*coR* I digestion and EGFP fragment were purified and MEGAscript RNAi Kit (Thermo Scientific, USA) was used in dsRNA synthesys according to the manufacturer's instructions.

For a short silencing experiment, 5 μg/g body dsRNA was injected into a single undifferentiated crayfish with an average weight of o.2 g and average body length of 2 cm by microsyringe (0–50 μL), which manufactured by Ningbo zhenhai sanai instrument factory. Cephalothoraxes of dsRNA-injected individuals were collected at two weeks after injection with dsEGFP or dsDsx, respectively. qRT-PCR was performed to investigate the expression level of *Cqdsx* and *Cq-IAG*.

Statistical Analyses

Statistical analyses were performed using SPSS software version 13.0. Data were expressed as mean ± SD (n=3) and statistical significance was determined by one-way ANOVA. Significance was set at $P < 0.05$.

Results

Cloning and Sequence Analysis of Full-length Cqdsx cDNA

A EST sequence containing DM domain from *C. quadricarinatus* RNA-seq data was identified. On this basis, the full-length cDNA sequence of *dsx* was obtained by merging the sequences of the 3’ and 5’-RACE products, designated as *Cqdsx*. Sequence analysis showed that the *Cqdsx* cDNA was 1271 bp in length, comprising a 155-bp 5’-untranslated region (5’-UTR), a 885-bp predicted open reading frame (ORF) encoding 294 amino acid polypeptides, and a 231-bp 3’-UTR. The *Cqdsx* cDNA sequence has a polyadenylation signal sequence (AATAAA) at the 3’ end, with predicted molecular mass of 32.7 kDa and isoelectric point of 6.12 (Fig 1). The complete sequence has been submitted to GenBank with the accession number MK342618.

Sequence Alignment and Phylogenetic Analysis

Protein domains analysis by InterPro revealed that the deduced amino acid sequence of Cqdsx protein contained a highly conserved DM domain shared by DM-domain gene family. As shown in Fig 2a, *D. melanogaster* and *B. mori* also found an oligomerization domain at the carboxy-terminus, which absent in crustaceans, including *C. quadricarinatus*, *Fenneropenaeus chinensis*, *Daphnia magna*. Multiple alignment of DM-domain coding sequence showed that *C. quadricarinatus* exhibited 50% identity to other species Dsx proteins (Fig 2b). Phylogenetic analysis of various Dsx proteins (Table 2) including crustaceans and insects was constructed using Neighbor-joining method of MEGA 5.0 software with 500 bootstrap replicates. The result showed that the phylogenetic tree could be divided into two major groups. Dsx proteins from crustaceans were clustered into one clade and others were clustered into another clade (Fig 2c).

Quantitative Expression of Cqdsx In Various Tissues and Different Developmental Stages

The transcript levels of *Cqdsx* gene from various tissues including gill, heart, hepatopancreas, muscle, intestine, ovary and testis were investigated by quantitative real-time RT-PCR. The results revealed that the *Cqdsx* mRNA was strongly expressed in gonads and little expression was observed in other tissues (Fig 3a). Moreover, the expression patterns of *Cqdsx* during embryonic and ovarian developmental stages were analyzed by qRT-PCR. The results showed that *w*ith the progression of embryonic development, the abundance of *Cqdsx* transcripts rapidly increased, reaching the maximum level at the prehatching-stage (Fig 3b). In addition, *Cqdsx* gene widely expressed in all examined ovary development stages and exhibited the higher transcript levels in the early stages (Fig 3c).

Localization of Cqdsx Transcripts In the Gonads of C. quadricarinatus

Cellular localization of *Cqdsx* transcripts in ovaries and testes of *C. quadricarinatus* were performed using in situ hybridization. Intensive hybridization signals from ovary were exclusively observed in oocytes and ovarian lamellae (Fig 4a), while in testis weak signals were detected in spermatocyte (Fig

4b). Obviously, these results were consistent with *Cqdsx* mRNA expression in gonads, which higher expressed in ovary and weakly expressed in testis.

Effect of Cqdsx dsRNA Injection On the Expression Levels of Cq-IAG

To further investigate the role of *Cqdsx* gene in sex determination and differentiation of *C. quadricarinatus*, RNAi assay was employed in this study via dsRNA injection into undifferentiated crayfish. Upon silencing of the *Cqdsx* gene, the transcription level was significantly reduced by >75% (P<0.01, Fig 5), suggesting dsRNA-mediated silencing of *Cqdsx* gene was successful. Interestingly, a nearly twice increase of the *Cq-IAG* transcripts was observed after knockdown of *Cqdsx* gene (Fig 5).

Discussion

As is known to us, the crustaceans usually represent significant sexual dimorphism (i.e., body size and growth rate) during the growth process [2, 18]. Therefore, it is of great value to elucidate its sex determination and differentiation mechanism to develop sex-control breeding. Many previous studies have shown that dsx gene, the key regulator of the most downstream of the sex regulation cascade, appears to have the longest time during sexual development. Currently, dsx homologous genes were identified in D. melanogaster and its related species [7, 10, 19, 20,], but further research is needed in crustaceans. In the present study, we reported the cloning and characterization of the Cqdsx gene and its primary function was analyzed in redclaw crayfish for the first time.

Here, only one isoform of Cqdsx gene was isolated from C. QUADRICARINATUS, which was consistent with the case in F. chinensis [13], while differed from insects containing several spliced variants. Sequence analysis showed that Dsx protein of C. QUADRICARINATUS AND F. chinensis had a highly conserved DM domain, but lacked of sex-specific OD2 domain that existing in insects [21] and D. magna [12]. It have been shown that the dsx gene mRNA precursor can be sexually spliced to produce a female- or male-specific RNA, which encodes female- and male-specific proteins Dsx^F and Dsx^M, respectively. Many insect species-related studies have revealed that both Dsx^F and Dsx^M regulate sex-specific morphologies directly via inhibiting or activating the expression of target genes [22, 23]. Since protein structure and alternative splicing of Cqdsx has an extremely significant difference compared with other non-decapoda species. Therefoce, further research is needed to understand the mechanism of sexual regulation of C. quadricarinatus.

The mRNA expression profiles of Cqdsx gene at various stages of embryos, ovarian development and adult tissues were analyzed by qRT-PCR. The obtained quantitative results of embryonic stages (Fig. 3b) implied that its possible function in the sex determination process, which was similar to the results from Bemisia tabaci [24] and F. chinensis [13]. In addition, tissues distribution analysis revealed that the unique distribution in gonads of Cqdsx gene was observed in C. quadricarinatus (Fig. 3a). The expression level in the ovary was significantly higher than that in the testis and other tissues, suggesting that Cqdsx was essential for ovarian differentiation. These finding were fully supported by the results of in situ

hybridization, which the Cqdsx transcripts was mainly detected in oocytesand weak signal in spermatocyte (Fig. 4). In summary, the expression pattern of Cqdsx in this study demonstrates that it may play an important role in the male sex determination process of C. quadricarinatus.

To further characterize the role of Cqdsx gene in the female sex determination, silencing of Cqdsx was performed by specific dsRNA injection. In crustaceans, an specific insulin-like gene (IAG) secreted by the androgenic gland (AG), has proved to be involved in inducing masculinity [25, 26]. In the current study, the expression analysis of Cq-IAG was examined in the Cqdsx knocked-down redclaw crayfish. Substantial change was observed on expression level of up-regulated in Cq-IAG after the Cqdsx gene silencing (Fig. 5). Considering the function of Cq-IAG in sex determination, we hypothesize that Cqdsx may participate in the regulation of sexual differentiation in C. quadricarinatus.

Conclusions

In summary, we isolated and characterized the full-length cDNA of Cqdsx in C. quadricarinatus for the first time. Expression profiles showed that Cqdsx mRNA was predominantly transcripted in the ovary, which indicated its role in ovarian differentiation. In RNAi assay, the expression level of Cq-IAG was significantly changed after knockdown of Cqdsx. These data may provide us a better understanding of sex determination in C. quadricarinatus.

Declarations

Acknowledgements

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Contributions

JZ and ZG designed the research. LC, JZ, YJ, and SL performed the experiments. JZ and LC analyzed the data. MC, LC and SC contributed reagents and animal materials. LC, JZ and ZG wrote the manuscript. All authors reviewed the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The gene sequences used for qRT-PCR analysis were available and download from the public database National Center for Biotechnology Information under the accession codes with MK342618. All data supporting the findings of our study can be found within the manuscript.

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Tables

Table 1 Primers used in this study.

Name	Sequences (5’-3’)	Tm (°C)	Purpose
5’RACE-1	CAGTTGTGATTTTCTCGGTAAGGGCAG	60	5’-RACE outer primer
5’RACE-2	TTGGCACACATACGACACCGTTGTTTT	58	5’-RACE inner primer
3’RACE-1	AAAACAACGGTGTCGTATGTGTGCCAA	58	3’-RACE outer primer
3’RACE-2	TACTGCCCTTACCGAGAAAATCACAAC	58	3’-RACE inner primer
RT-*dsx*-F	TGGCACTCTGGTCCAGGAAGG	58	qRT-PCR
RT-*dsx*-R	CCGTCGTCGTCATCAGCAGTAG	59	qRT-PCR
18S-F	CTGAGAAACGGCTACCACATC	54	qRT-PCR
18S-R	GCCGGGAGTGGGTAATTT	50	qRT-PCR
Probe-*dsx*-F	GCTCTAGAATGGCTATGGCGCCTAAGAA	61	probesynthesis
Probe-*dsx*-R	GGAATTCAGTACCACTTGTGGCCCTTC	61	probe synthesis
dsRNA-*dsx*-F	GAATTCTAATACGACTCACTATAGGGATGGCTATGGCGCCTAAGAA	68	dsRNA synthesis
dsRNA-*dsx*-R	AAGCTTTAATACGACTCACTATAGGGTCACCGTGGTACTGATGAAC	68	dsRNAsynthesis
Cq-IAG-F	ACTGTGAACAGTTGGAGGACGGA	63	qRT-PCR
Cq-IAG-R	ATGGAAGGCGCTGGAAAGCCATG	71	qRT-PCR

Table 2 Dsx proteins used for construction of the phylogenetic tree

Species	GenBank accession No.	Species	GenBank accession No.
Daphnia pulex	AGJ 52190.1	*Daphnia galeata*	BAM 33609.1
Fenneropenaeus chinensis	JQ965255	*Apis mellifera*	ABW99105.1
Anopheles gambiae	AAZ78362.1	*Drosophila melanogaster*	NM_169202
Nasonia vitripennis	ACJ65508.1	*Bombyx mori*	NP_001036871
Tribolium castaneum	AFQ62105.1	*Onthophagus taurus*	AEX92938.1
Daphnia magna	JQ965255	*Bactrocera tryoni*	AAB99948
Bactrocera oleae	CAD 67987	*Cherax quadricariratus*	MK342618
Trypoxylus dichotomus	BAM93340.1		

Figures

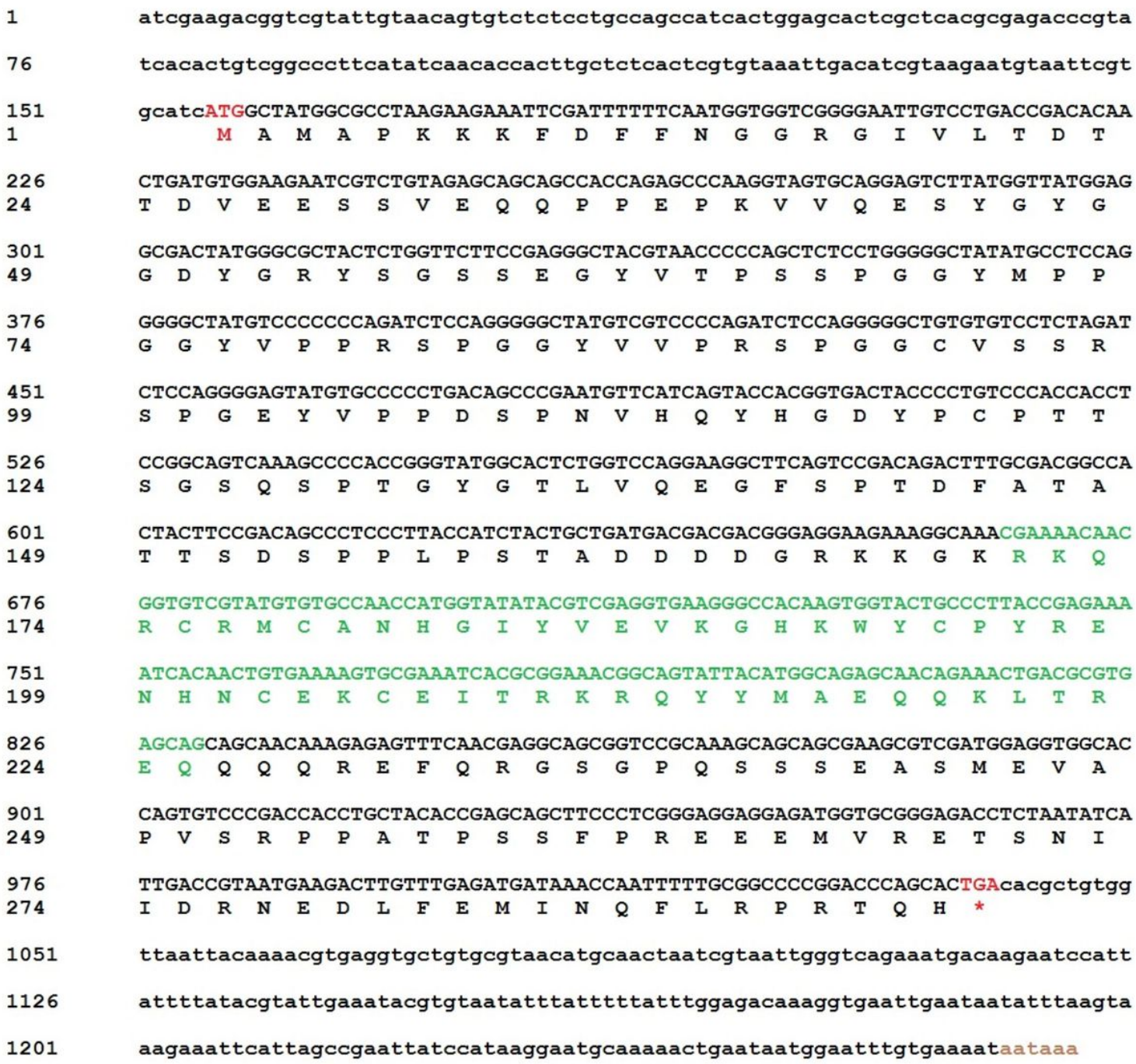

Figure 1

Nucleotide sequence and deduced amino acid sequence of Cqdsx. The start codon and stop codon are shown in red background, the polyadenylation signal sequence (AATAAA) is shown in orange, and the predicted DM domain is shown in green.

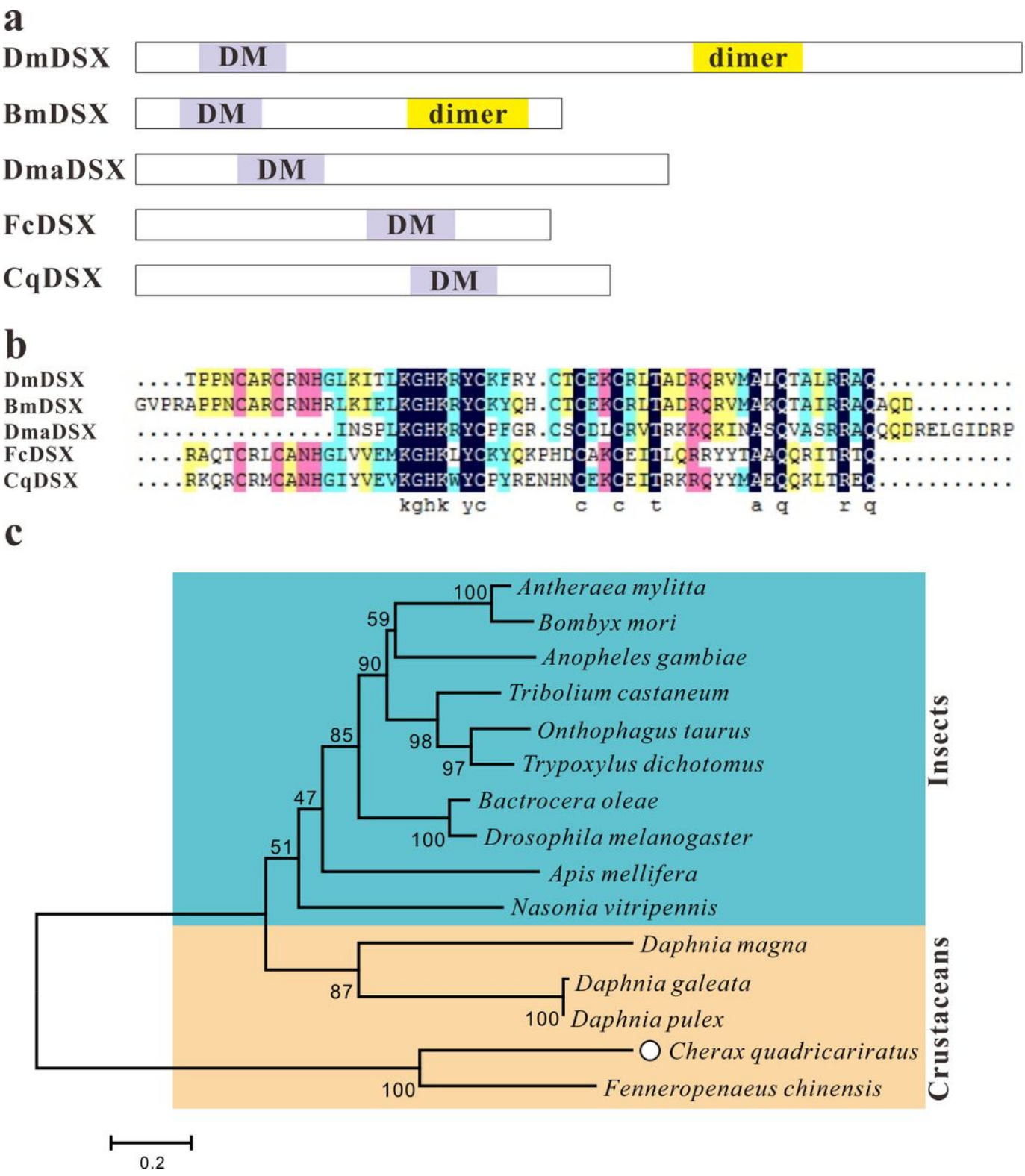

Figure 2

Comparision and phylogenic analysis of CqDsx protein with other homologous amino acid sequence. (a) The second structure prediction of Dsx proteins using InterProScan software. Dm D. melanogaster, Bm B. mori, Dma D. magna, Fc F. chinensis, Cq C. quadricarinatus. (b) Multiple alignment of DM-domain coding sequence. (c) Neighbor-joining tree of Dsx protein generated by MEGA 5.0 software with bootstrap values of 500 replicates. CqDsx is shown in circle.

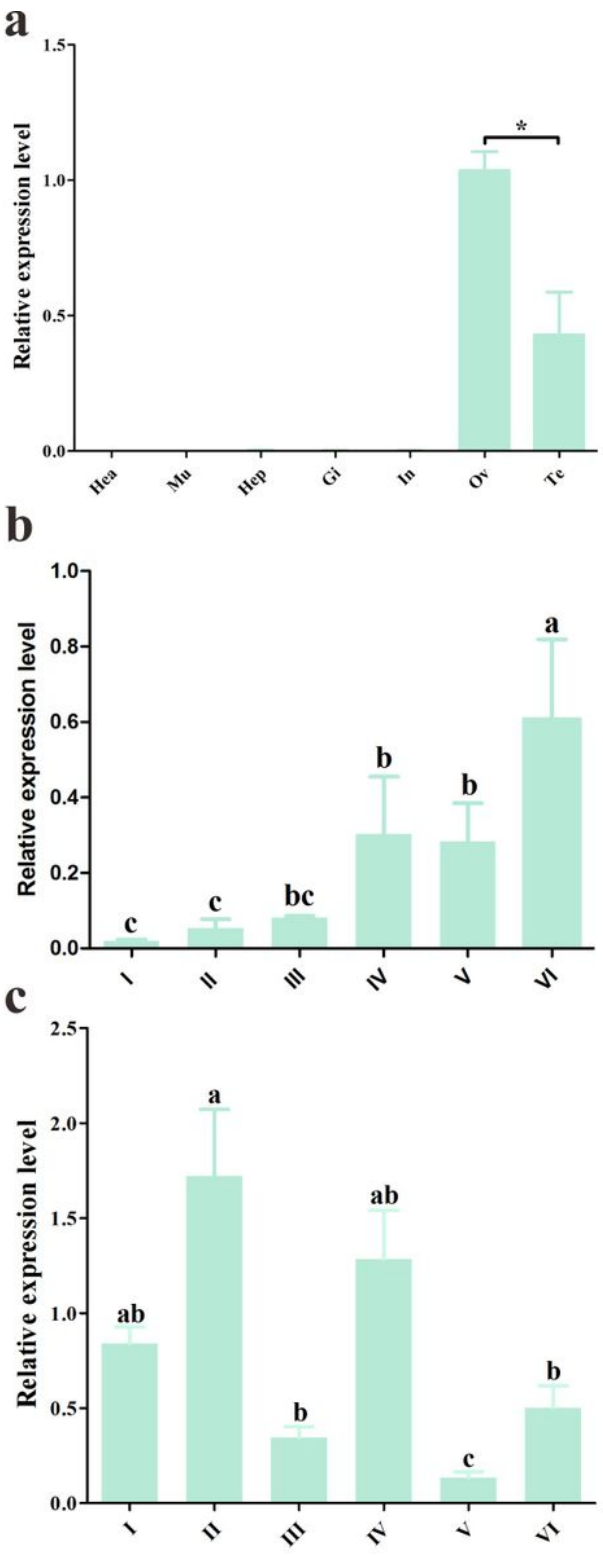

Figure 3

Expression profiles of Cqdsx in different tissues (a), embryonic (b) and ovarian development stages (c) of C. quadricarinatus by quantitative real-time RT-PCR. (a) Hea heart, Mu muscle, Tg thoracic ganglia, Hep hepatopancreas, Gi Gill, In intestine, Te testis, Ov ovary. (b) I fertilized eggs, II cleavage and blastula, III gastrula, IV nauplius, V eye pigments forming, VI prehatching. (c) I-VI indicate stage I to stage VI of ovarian development period. The 18S-rRNA gene was used as control. Data are expressed as the mean

fold change (means ± SD, n=3). Statistical significance was calculated by Tukey multiple comparison tests and Student's t test, indicated by lettered bars ($p < 0.001$).

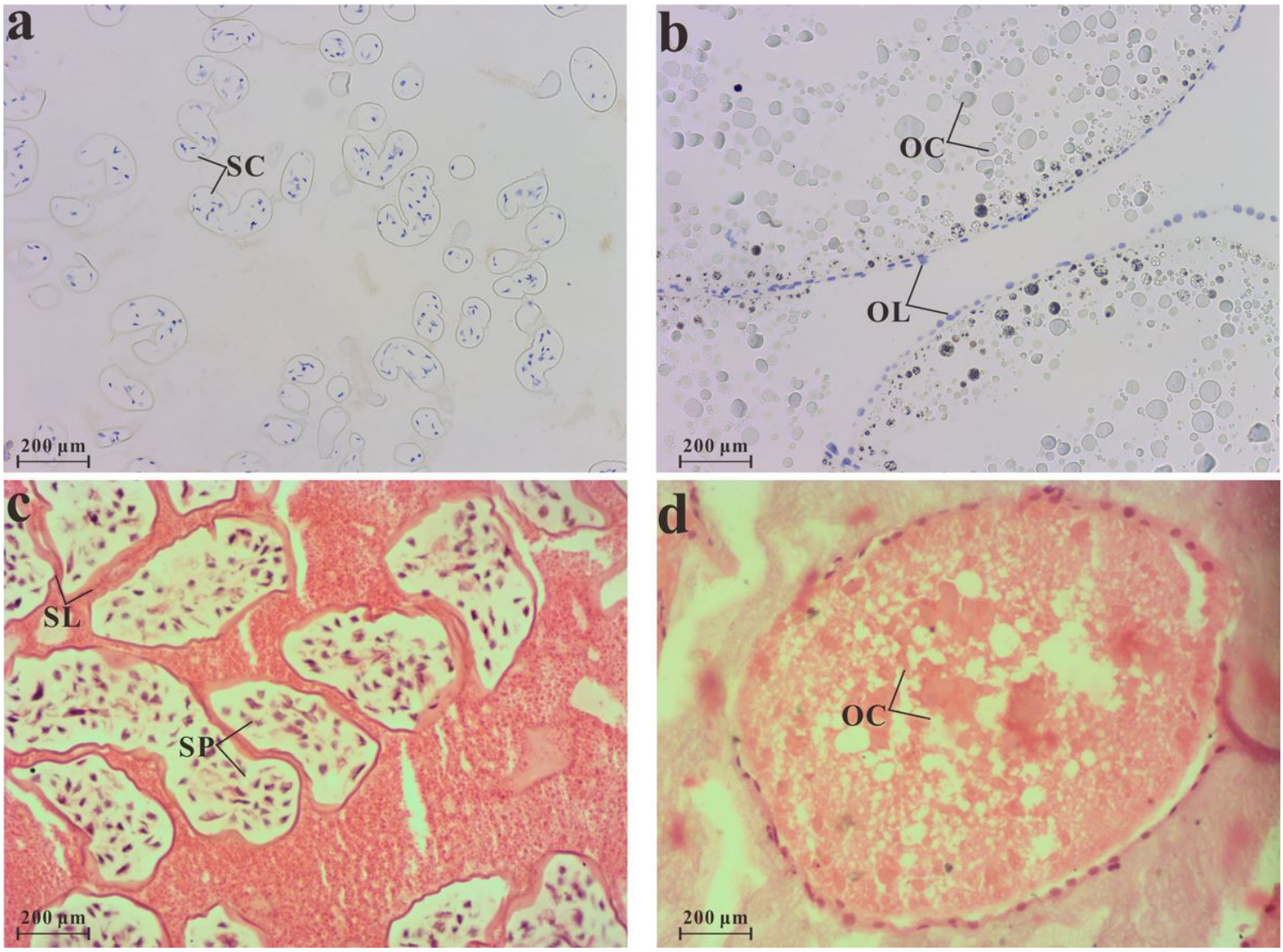

Figure 4

In situ localization of Cqdsx mRNA in gonads of C. quadricarinatus. Testis (a) and ovary (b) in situ hybridization using antisense RNA probe of Cqdsx. HE staining (of normal testis (c) and ovary (d) section. Tissue sections (thickness 6 μm) were stained with hematoxylin and eosin. SC spermatocyte, SL seminiferous lobules, SP spermatid, OL ovarian lamellae, and OC oocyte. A purple color corresponds to a positive signal. The scale bars are 200 μm.

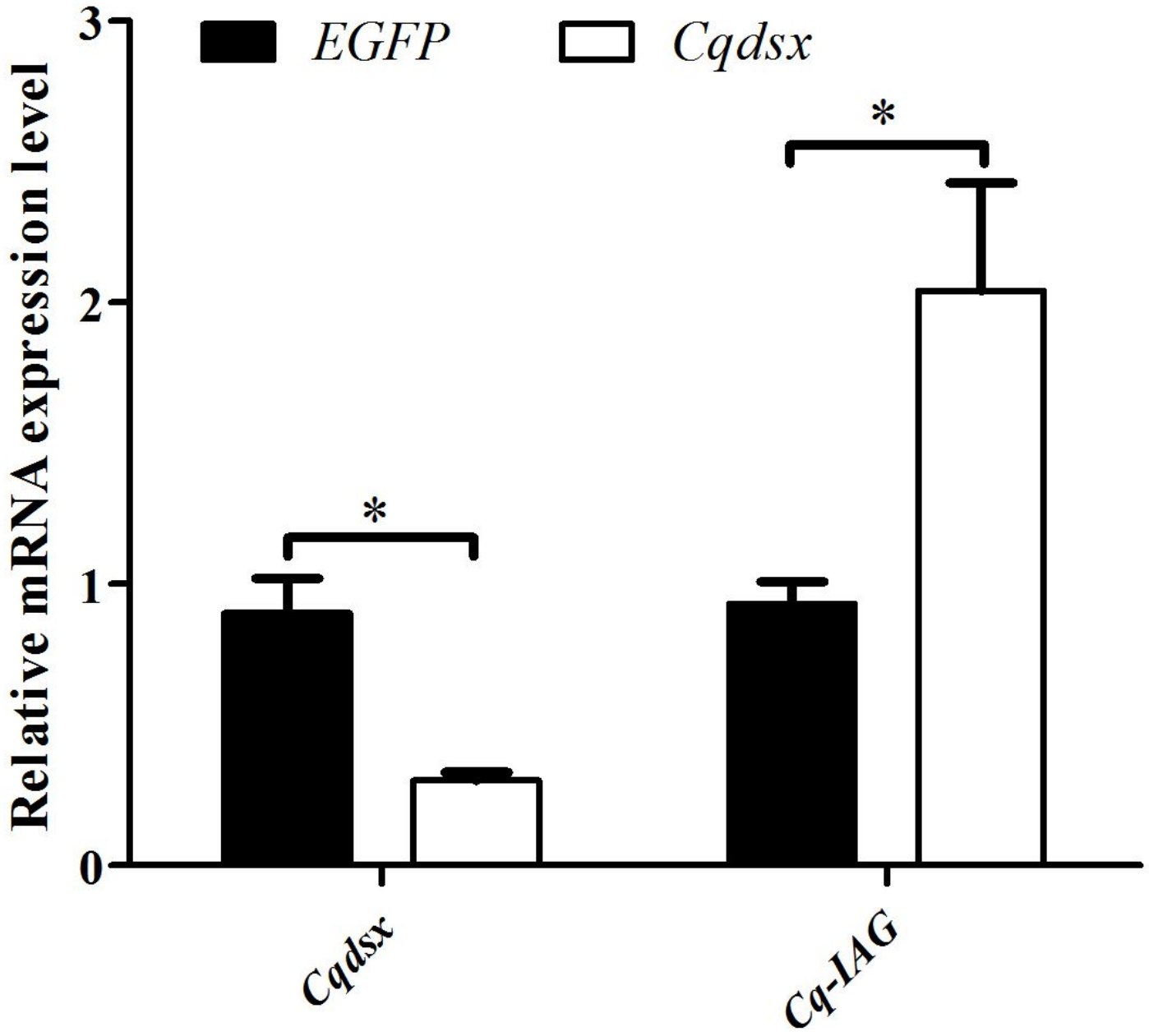

Figure 5

Relative expression of Cqdsx and CqIAG after injection dsRNA. qRT-PCR examined mRNA levels of Cqdsx genes as well as male- or female-reproduction related genes in cephalothoraxes after Cqdsx RNAi. EGFP, treated with dsEGFP and used as RNAi control; Cqdsx, treated with dsCqdsx. Three individuals were pooled as one sample. Three replicates were used for analysis. Results are expressed as mean±SEM and significance of comparison is defined as $P<0.05$ (*) by Student's t tests.