Transcriptome analysis of different developmental stages of Daphnia similoides sinensis and screening of male sex determination candidate genes

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Research Article

Keywords: Daphnia similoides sinensis, transcriptome sequencing, male sex determination, differentially expressed genes, candidate gene

Posted Date: March 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1438431/v1

Abstract

Background: In the life history of *Daphnia*, the reproductive mode of parthenogenesis and sexual reproduction alternate in aquatic ecosystem, which are often affected by environmental and genetic factor. Although several functional genes on reproductive transition of *Daphnia* had been determined, molecular mechanism on the reproductive mode of *Daphnia* are still not known well, including differentially expressed genes in different developmental stages.

Results: In this study, four developmental stages, juvenile female (JF), parthenogenetic female (PF), sexual female (SF) and male (M), of *D. similoides sinensis* were performed for transcriptome sequence, and candidate genes related to male sex determination were screened. A total of 110437 transcripts were obtained and assembled into 22996 unigenes. In the four developmental stages (JF, PF, SF and M), the number of unique unigenes is respectively 2863, 445, 437 and 586, and the number of common unigenes is 9708. The differentially expressed genes (DEGs) between male and other three female stages (M vs JF, M vs PF and M vs SF) were obtained. GO gene enrichment analysis showed that the up-regulated genes in male were mainly enriched in hydrolase activity and peptidase activity.

Thirty-six candidate genes related to male sex determination in male were significantly higher expression than those in the other three stages, including one *Doublesex* (*Dsx*) gene, one laminin gene, five trypsin genes and one serine protease genes, and one chitin synthase gene and two chitinase genes. In addition, in *D. similoides sinensis* male, the relative expression levels of two genes (*Dsx1, antp*) related to male sex determination observed in other *Daphnia* species were also significantly higher than those in the other three stages.

Conclusions: Our results showed that thirty-six candidate genes may involve in sex differentiation of *D. similoides sinensis* male, and it will provide a reference for further exploring the functional genes related to sex determination mechanism in *Daphnia* species. Moreover, according to previous investigations, we thought that the expression level of functional genes may be related to the development stages of organisms, and may be also affected by different *Daphnia* species.

Background

In the life history of *Daphnia*, parthenogenesis and sexual reproduction often alternate in aquatic ecosystem, which is affected by environmental (e.g. food, predation, photoperiod) and genetic (e.g. genotype) factors together [1]. Under suitable environmental conditions, they only produce female offspring by parthenogenesis. However, when environmental conditions deteriorate (such as fish predation, food shortage and higher population density), *Daphnia* species will transfer from parthenogenesis to sexual reproduction, producing male and sexual female, and then mate and fertilize, forming resting eggs [2-5]. Resting eggs can survive in lake sediments for several decades [6], and then hatch and form new populations in suitable conditions. Some investigations have indicated that *Daphnia*

is an ideal model organism in studying ecology, environmental toxicology and evolutionary biology [7, 8]. However, molecular mechanisms on male sex determination of *Daphnia* are still not well-known.

Previous investigations have shown that several genes play key roles in reproductive regulation and male sex determination in cladocera. DM domain gene has been identified as a sex-determining gene in many species [9, 10]. Kato et al [11] found that the DM domain gene DMRT93B, which was only expressed in testis, may regulate the differentiation and maintenance of *D. magna* testis. Schwarzenberger and Von Elert [12] observed that the expression level of *Hox* gene *antennapedia* (*antp*) in the first antennae of *D. magna* male adults was significantly higher than that in the first antennae of female adults, suggesting that *antp* may be involved in the molecular pathway inducing the male phenotype of *Daphnia*. Kato et al [13] found that knock-down *Dsx1* in male embryos of *D. magna* resulted in the production of female traits including ovarian maturation, whereas ectopic expression of *Dsx1* in female embryos resulted in the development of male-like phenotypes. Therefore, it suggests that *Dsx1* is a key regulator of male phenotype in *D. magna*. Chen et al [14] found that a *Transformer* (*Tra*) gene was crucial in the sex determination of *Daphnia pulex*. In *Daphnia carinata*, dephosphorylation of *Tra* gene may be the trigger for transformation from females to male [15]. Mohamad Ishak et al [16] observed that the expression level of Fushi tarazu factor-1 (*Ftz-F1*) in male embryos of *D. magna* was significantly higher than that in female embryos, suggesting that *Ftz-F1* may be involved in the male producing in response to environmental stimuli. Toyota et al [17] found that expression of the juvenile hormone acid O-methyltransferase (*JHAMT*) gene increased significantly before the MF-sensitive period (MF: methyl farnesoate, putative juvenile hormone, in daphnids) for male production in *D. pulex*, suggesting that *JHAMT* may induce male offspring by regulating MF synthesis. However, the screening of genes related to male sex determination of *Daphnia* species is incomplete, and even was not reported in *D. similoides sinensis*.

In general, sex determination of *Daphnia* is a complex process. The specific regulatory mechanism and functional genes in this process are not clear. Our goals are to compare the transcriptome of *D. similoides sinensis* at four developmental stages (i.e. juvenile female, parthenogenetic female, sexual female and male), and to screen the candidate genes related to male sex determination from DEGs. Our study will provide a reference for further exploring the functional genes related to sex determination mechanism in *Daphnia* species

Methods

D. similoides sinensis and *Scenedesmus obliquus* culture

D. similoides sinensis employed in the experiment were collected from Lake Chaohu in Anhui Province, China. Parthenogenetic females were cultivated under a 12 h light /12 h dark photoperiod at 25±1℃ with a light intensity of 2500 lx, and fed with 40 mg L^{-1} of *S. obliquus* (wet weight). *S. obliquus* was obtained from the Freshwater Algae Culture Collection at Institute of Hydrobiology, Chinese Academy of Sciences, and cultured in BG-11 medium at 25±1℃ with a 12 h light/12 h dark photoperiod.

Juvenile female (JF), parthenogenetic female (PF), sexual female (SF) and male (M) collection

In one experiment, 20 *D. similoides sinensis* mothers were employed. Each mother was respectively cultured in a 50 ml beaker with 40 ml culture medium. The culture medium was replaced every two days. The culture medium was the filtered and aerated tap water. Offspring (birth time <12h) produced by *D. similoides sinensis* mother were collected as juvenile female samples (JF), and each juvenile female sample was about 500 individuals. These neonates produced by the mother were removed in time from beakers during the experiment.

In another experiment, 60 *D. similoides sinensis* mothers were employed. Each 10 mothers were placed in a 250 ml beaker with 200 ml culture medium, 6 replicates were conducted. The culture medium was replaced every two days. After 2-3 weeks, sexual females and males of *D. similoides sinensis* could be observed when population density became higher. The parthenogenetic females (PF), sexual females (SF) and males (M) were selected under the microscope. About 50 parthenogenetic females and 50 sexual females were respectively collected as PF sample and SF sample. Male juveniles were collected and cultured in a 100 mL beaker with 80 mL culture medium for two weeks. Each male sample (M) contained about 60 male individuals.

JF, PF, SF and M samples were put into EP tubes, respectively. These samples were immediately frozen in liquid nitrogen and stored at -80℃. In this study, two replicates of JF, PF, SF and M samples were collected for transcriptome sequencing, respectively.

RNA isolation and cDNA library construction

Total RNA was respectively extracted from samples at four developmental stages of *D. similoides sinensis* (juvenile females, parthenogenetic females, sexual females and males) using TRIzol reagent. RNA degradation and contamination was monitored using 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

A total of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV reverse transcriptase (RNase H^-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. In order to select cDNA fragments of

preferentially 250~300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15min, followed by 5 min at 95°C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified with AMPure XP system and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing

The clustering of the index-coded samples was performed with a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

De novo assembly of short reads and gene annotation

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. Clean data (clean reads) for the JF, PF, SF and M samples were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. Simultaneously, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. Transcriptome assembly was accomplished based on the left.fq and right.fq using Trinity [18] with min_kmer_cov set to 2 by default, and all other parameters set default. The resulting sequences were named unigenes. The unigenes were annotated by BLASTx searching in NCBI non-redundant (Nr), Swiss-Prot, KEGG, PFAM and KOG and mapped to NCBI Nt database by BLASTn. Functional annotation by Gene Ontology (GO) terms was analyzed by using Blast2GO (http://www.balst2go.org/) software [19]. GO functional classification for unigenes was analyzed using WEGO software [20]. The similarity searches of unigenes were performed using the NCBI-BLAST network server (http://blast.ncbi.nlm.nih.gov/).

Differential expression genes and GO enrichment analysis

Differential expression analysis between two developmental stages (JF vs PF, PF vs SF and M vs PF) of *D. similoides sinensis* was performed using the DEGseq 2 package. *P*-value was adjusted using *Q-value* [21]. *Q-value*<0.05 and $|\log_2(\text{fold change})|>1$ were set as the threshold for significantly differential expression. GO enrichment analysis of DEGs was implemented by the GOseq packages based on Wallenius' non-central hyper-geometric distribution [22].

Validation of DEGs using Real Time-PCR

Total RNA was extracted using TRIzol reagent (TaKaRa, Dalian, China). The ultramicro-spectrophotometer (MD2000D, Biofuture, UK) was used to assess sample purity and RNA concentration. RNA was reversely transcribed by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China).

RT-qPCR was performed with 76 genes, which were selected from top 30 up-regulation DEGs in M vs JF, M vs PF and M vs SF. The qPCR primers were designed using Beacon Designer 7.9 (PREMIER Biosoft International, Palo Alto, CA, USA) and listed in Table S1. *DsimGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as the reference gene and also listed in Table S1. The qPCR was performed in a LightCycler® 96 (Roche Diagnostics Gmbh, Basel, Switzerland) using a mixture of 5.0 μL AceQ qPCR SYBR Green Master Mix (Vazyme, China), 0.2 μL of each primer, 1.0 μL of sample cDNA and 3.6 μL of RNase Free dH_2O. The amplification step was executed using a degeneration step at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 60 s. The melting curve was employed to detect a single primer-specific peak, using 93℃ for 30 s and 60℃ for 45 s. All reactions were run in triplicate. The relative quantification results were analyzed using the Ct method ($2^{-\Delta\Delta C}T$) [23] .

Statistical analysis

Statistical analysis was executed by SPSS 20.0 software. Significant differences of relative mRNA expression level between two developmental stages (M vs JF, M vs PF and M vs SF) were analyzed using multiple comparisons Turkey (HSD). All data were shown as mean±SEM in this study.

Results

Transcriptome sequencing, sequence assembly and annotation

Among transcriptome sequencing of four developmental stages (JF-juvenile female, PF-parthenogenetic female, SF-sexual female and M-male) of *D. similoides sinensis*, 110437 transcripts were obtained, with a total length of 321269424 bp and an average length of 2909 bp. Moreover, 22996 unigenes were obtained, with a total length of 44512763 bp, an average length of 1936 bp and a N_{50} length of 4265 bp (Table 1). Compared with several common databases through BLASTx program, the most unigenes annotated to Nr database (13512, accounting for 58.75% of the total unigenes), followed by PFAM (10659, accounting for 46.35%) and GO (10659, accounting for 46.35%) (Table 2).

Table 1 Assembly analysis of transcriptome from four developmental stages of *D. similoides sinensis*

	Transcripts	Unigenes
200-500bp	18137	6898
500-1000 bp	17791	6040
1k-2000 bp	20536	3619
>2000 bp	53973	6439
Total number	110437	22996
Total length	321269424	44512763
Mean length	2909	1936
N_{50} length	5000	4265

Table 2 Summary statistics on functional annotation of unigenes in *D. similoides sinensis* tanscriptome

	Number of Genes	Percentage (%)
Nr	13512	58.75
Nt	3957	17.20
KEGG	6211	27.00
Swiss-Prot	9354	40.67
PFAM	10659	46.35
KOG	6472	28.14
GO	10659	46.35

Homology analysis

The homologous sequences of *D. similoides sinensis* unigenes were matched in Nr database. The relative species with higher homologous sequences were *D. magna* (70.5%), followed by *D. pulex* (10.0%), *Tetrahymena thermophila* (1.9%), *Pseudocohnilembus persalinus* (1.1%), *Ichthyophthirius multifiliis* (0.9%) and other (15.6%) (Fig. 1).

Differentially expressed genes

The number of specific unigenes in JF, PF, SF and M developmental stages were 2863, 445, 437 and 586, respectively. There were 9708 common unigenes in four developmental stages (Fig. 2). The differentially expression genes (DEGs) between the two stages were determined by comparing the genes obtained in male with genes in the other three female stages. In differentially expressed genes, the number of up-

regulated genes and down-regulated genes were 2230 and 2340 in M vs JF, 2425 and 1933 in M vs PF, and 1473 and 1382 in M vs SF, respectively (Fig. 3).

Gene ontology annotation

To analyze the functions of these DEGs, we conducted 4670 DEGs in M vs JF, 4358 DEGs in M vs PF and 2855 DEGs in M vs SF by using the GO enrichment system (*Q-value* <0.05). In M vs JF, up-regulated genes were mainly concentrated in protein metabolic process (506) and hydrolase activity (493), and the down-regulated genes were mainly concentrated in macromolecule biosynthetic process (429), cellular macromolecule biosynthetic process (426), cellular nitrogen compound biosynthetic process (421) and gene expression (418). In M vs PF, the up-regulated genes were mainly concentrated in hydrolase activity (503), and the down-regulated genes were mainly concentrated in nucleic acid binding (370) and gene expression (342). In M vs SF, the up-regulated genes were mainly concentrated in hydrolase activity (132), peptidase activity of acting on L-amino acid peptides (128) and proteolysis (119), and the down-regulated genes were mainly concentrated in nuclear acid binding (233) (Fig. 4).

Candidate genes related to male sex determination

In order to screen candidate genes related to male sex determination of *D. similoides sinensis*, a total of 76 genes were respectively obtained from the top 30 up-regulated DEGs in M vs JF, M vs PF and M vs SF. qPCR analysis showed that the relative expression levels of 36 genes in male (M) were significantly higher than those in the other three female stages (JF, PF and SF) (P<0.05), suggesting that these genes may participate in the male sex determination of *D. similoides sinensis* (Fig. 5, Table 3). Among them, there are 11 known genes, including one *Doublesex* gene (Cluster-5789.12340), one laminin gene (Cluster-5789.8159), one chitin synthase gene (Cluster-5789.11830), two chitinase genes (Cluster-5789.5191 and Cluster-5789.7417), five trypsin genes (Cluster-5789.9553, Cluster-5789.3677, Cluster-5789.9554, Cluster-5789.11655 and Cluster-5789.7668) and one serine protease gene (Cluster-5789.2039). The other 25 genes were uncharacterized (Table 3).

In addition, some previous known genes (*Dsx1*, *Tra*, *antp* and DMRT93B) related to male sex determination in other *Daphnia* species appeared also in the differentially expressed genes of *D. similoides sinensis* (Fig. 6). The expression levels of *Dsx1* in male (M) was significantly (P<0.05) higher than that in the other three stages (JF, PF and SF), and the expression level of *antp* in male (M) was significantly (P<0.05) higher than those in both PF and SF whereas it was significantly lower than that in JF.

Table 3 Thirty-six differentially expressed genes related to male sex determination in *D. similoides sinensis*

Gene ID	Nr Description	*q-value*
Cluster-5789.12340	*Doublesex2*	1.31E-175
Cluster-5789.8159	laminin subunit gamma-3	3.05E-79
Cluster-5789.5191	chitinase 5	1.81E-210
Cluster-5789.7417	chitinase 8	1.0536E-284
Cluster-5789.11830	chitin synthase 2	2.83E-298
Cluster-5789.2039	serine protease ami-like	6.7587E-231
Cluster-5789.9553	trypsin-7-like protein	0
Cluster-5789.3677	putative trypsin-7	2.1334E-252
Cluster-5789.9554	putative trypsin-7, partial	2.1475E-205
Cluster-5789.11655	trypsin alpha-like	5.81E-235
Cluster-5789.7668	trypsin-like isoform X1	9.1381E-73
Cluster-5789.2410	uncharacterized protein APZ42_028762	2.48E-71
Cluster-5789.11844	uncharacterized protein LOC116923216 isoform X2	1.00E-77
Cluster-5789.11654	uncharacterized protein APZ42_030656	1.96E-46
Cluster-5789.11701	uncharacterized protein APZ42_020061	0
Cluster-5789.1218	uncharacterized protein LOC116919217	7.5696E-109
Cluster-5789.11046	uncharacterized protein LOC116919081	1.26E-220
Cluster-5789.6490	uncharacterized protein APZ42_017126	3.32E-214
Cluster-5789.11774	uncharacterized protein APZ42_015939	1.63E-292
Cluster-5789.1251	uncharacterized protein APZ42_031730	3.07E-256
Cluster-5789.1931	hypothetical protein DAPPUDRAFT_299805	6.35E-232
Cluster-5789.2216	uncharacterized protein APZ42_016906	3.73E-230
Cluster-5789.11766	uncharacterized protein APZ42_018760	7.8031E-166
Cluster-5789.2177	uncharacterized protein LOC116920378	2.7322E-165
Cluster-5789.11865	uncharacterized protein APZ42_014845	6.39E-185
Cluster-5789.6049	uncharacterized protein APZ42_018439	7.96E-127
Cluster-5789.1615	uncharacterized protein LOC116917111	2.39E-108
Cluster-5789.9163	uncharacterized protein LOC116927871	5.10E-107

Cluster-5789.11867	uncharacterized protein LOC116922766	8.63E-104
Cluster-5789.9162	uncharacterized protein LOC116927870	1.30E-95
Cluster-5789.11788	uncharacterized protein LOC116927218	4.68E-92
Cluster-5789.697	uncharacterized protein LOC116918656	1.47E-89
Cluster-5789.1257	uncharacterized protein APZ42_024939	6.62E-82
Cluster-5789.11457	uncharacterized protein LOC116929377 isoform X1	1.26E-81
Cluster-5789.1085	uncharacterized protein APZ42_033498	1.40E-74
Cluster-5789.11669	uncharacterized protein LOC116935274	1.6049E-220

Discussion

Usually, under worse conditions (such as fish predation, unsuited temperature and photoperiod), *Daphnia* transforms from parthenogenesis to sexual reproduction, producing male and sexual female, which mate and form resting eggs [24-26]. Previous studies have shown that several genes (*Dsx*, *antp*, *Tra* and DMRT93B) could play important roles in the male sex determination of *Daphnia*[11-14]. *Doublesex* (*Dsx*) gene is an important sex regulatory gene, which was first found in *Drosophila* [27], and has been widely studied in cladocera. Kato et al [13] found that knock-out *Dsx1* in male embryos of *D. magna* will lead to the production of female characteristics including ovarian maturation, while ectopic expression of *Dsx1* in female embryos will lead to the development of male-like phenotype, and thought that *Dsx1* is a key regulator of male phenotype in *D. magna*. In *D. carinata*, *Dsx1* and *Dsx2* may be also involved in the sex differentiation. Usually, *Dsx* contains two conserved domains: one is Dsx/Mab-3 (DM) domain at the N-terminal, and the other is oligomeric domain at the C-terminal [28]. DM-domain plays an important role in the sex determination of vertebrates [10, 29]. Kato et al [11] found that DM-domain gene DMRT93B in *D. magna* was only expressed in testis. In this study, the expression of *Doublesex2* (Cluster-5789.12340) in male was significantly (P<0.05) higher than those in the three females (Juvenile female, parthenogenetic female and sexual female), suggesting that *Doublesex2* may also play an important role in male sex determination in *D. similoides sinensis*. However, *DMRT93B* did not express in the four developmental stages of *D. similoides sinensis*.

For other arthropods, the formations of differential morphology of legs and antennae were regulated by the Hox gene *antp* [30, 31]. Schwarzenberger and Von Elert [12] observed also that the *antp* expression level in the first antennae of *D. magna* male adults was significantly higher than that in the first antennae of female adults, and thought that *antp* may be involved in the molecular pathway of inducement to male phenotype of *Daphnia*. In this study, the expression level of *antp* in male was significantly (P<0.05) higher than those in females (parthenogenetic female and sexual female), suggesting that *antp* may be responsible for male sex determination of *D. similoides sinensis*. Moreover, the expression of *Tra* in *D. carinata* male was significantly higher than those in both parthenogenetic female and sexual female [15], which was also thought to be responsible for male sex determination of *Daphnia*. However, in this study,

the expression level of *Tra* in *D. similoides sinensis* male was significantly lower than that in parthenogenetic female, indicating that *Tra* may not play an important role in male sex determination of *D. similoides sinensis*. Therefore, it is likely that the expression level of functional genes may be related to the development stage of organisms, and is also affected by different *Daphnia* species.

Laminin (Ln) is a component of the basement membrane of male and female gonads in the frog *Rana rugosa* [32]. The synthesis of basement membrane is essential for sex differentiation of embryonic mammalian gonads [33]. The expression of laminin alpha 1 (*LAMA1*) in male and female bovine embryos showed sexual dimorphism [34]. Fröjdman et al [35] inferred that Ln alpha 5 chain is an early molecular marker of sexual differentiation in rat, which may be regulated by the testis-determining factors. In this study, the relative expression level of laminin subunit gamma-3 (Cluster-5789.8159) in male was significantly higher than those in the three females, suggesting that it may affect male sex differentiation through promoting the development of male gonads in *D. similoides sinensis*.

Chitin is the second abundant polysaccharide in nature, and it is the main component of fungal cell wall and exoskeleton of arthropod. Chitin is synthesized by chitin synthase and degraded by chitinase (Cht) to maintain the sustainable growth and development of organisms [36]. The chitin content in some male insects is significantly higher than that in female insects [37]. The expression levels of Cht 4 and Cht 4-1 in *Phenacoccus solenopsis* males were significantly higher than those in females [38]. Moreover, the Cht 4 gene in *Nilaparvata lugens* was only highly expressed in reproductive organs of adult male [39]. In this study, the expression levels of chitin synthase (Cluster-5789.11830) and chitinase (Cluster-5789.5191, Cluster-5789.7417) genes in *D. similoides sinensis* male were all significantly higher than those in the three females, suggesting that they may be related to male sex determination.

In summary, in this study, 36 candicate genes were screened to be responsible for male sex determination of *D. similoides sinensis*. In order to distinguish them, the functional identification of these candicate genes should be developed and determined in the future.

Conclusions

In this study, transcriptome sequences of the four developmental stages (JF-juvenile female, PF-parthenogenetic female, SF-sexual female and M-male) in *D. similoides sinensis* were investigated, and candidate genes related to male sex determination were screened (M vs JF, M vs PF and M vs SF). 110437 transcripts and 22996 unigenes were obtained. The number of specific ungenes in the four developmental stages (JF, PF, SF and M) were respectively 2863, 445, 437 and 586, with a common unigenesof 9708. In DEGs, the number of up-regulated and down-regulated genes were 2230 and 2340 in M vs JF, 2425 and 1933 in M vs PF, and 1473 and 1382 in M vs SF, respectively.

36 candidate genes related to male sex determination of *D. similoides sinensis* were obtained through screening from the top 30 up-regulated differentially expressed genes in M vs JF, M vs PF and M vs SF, and the expression levels of these genes in male were significantly higher than the other three females. Among these genes, there are 11 known genes, including one *Doublesex* gene (Cluster-5789.12340) which

was found to involve in sex differentiation of other *Daphnia* species, one laminin gene (Cluster-5789.8159) which possibly related to the development of male gonads in *D. similoides sinensis*, five trypsin genes (Cluster-5789.9553, Cluster-5789.3677, Cluster-5789.9554, Cluster-5789.11655 and Cluster-5789.7668) and one serine protease gene (Cluster-5789.2039) which may be related to sperm activation, and one chitin synthase gene (Cluster-5789.11830) and two chitinase genes (Cluster-5789.5191 and Cluster-5789.7417) which showed sexually dimorphic expression in *D. similoides sinensis*. In addition, some known genes (*Dsx*, *Tra*, *antp* and DMRT93B) related to male sex determination in other *Daphnia* species appeared also in differentially expressed genes of four developmental stages in *D. similoides sinensis*. The screening of the candidate genes will provide a reference for the identification of functional genes in *Daphnia* species and the molecular regulation mechanism of sex determination in cladocera.

However, some results (e.g. *Tra* and DMRT93B) in this study were inconsistent with previous investigations, suggesting that the expression level of functional genes may be related to the development stage of organisms, and may be also affected by different *Daphnia* species.

Abbreviations

JF: juvenile female; PF: parthenogenetic female; SF: sexual female; M: male; DEGs: differentially expressed genes; GO: Gene Ontology; *Dsx: Doublesex*; *Tra*: *Transformer*, *antp*: *antennapedia; Ftz-F1*: Fushi tarazu factor-1; JHAMT: juvenile hormone acid O-methyltransferase; MF: methyl farnesoate; RT-qPCR: Quantitative real-time polymerase chain reaction; *DsimGAPDH*: glyceraldehyde-3-phosphate dehydrogenase; Ln: Laminin; *LAMA1*: laminin alpha 1; Cht: chitinase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw RNA-Seq data used in this study have been deposited in the Nation Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database under the accession number GSE197943 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197943).

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Natural Science Foundation of China (No. 31370470, 31870451 and 32001155).

Authors' contributions

All authors conceived the main ideas and participated in shaping this research project. Daogui Deng is the project leader. Ziyan Wang conducted experiments and wrote the manuscript. Feiyun Zhang assisted experiments and analyzed the data. All authors have read and approved the final manuscript.

Acknowledgments

We thank Dr Zhang Yanan for his help in gene analysis.

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Figures

Figure 1

Percentage of homologous hits of *D. similoides sinensis* unigenes to other species

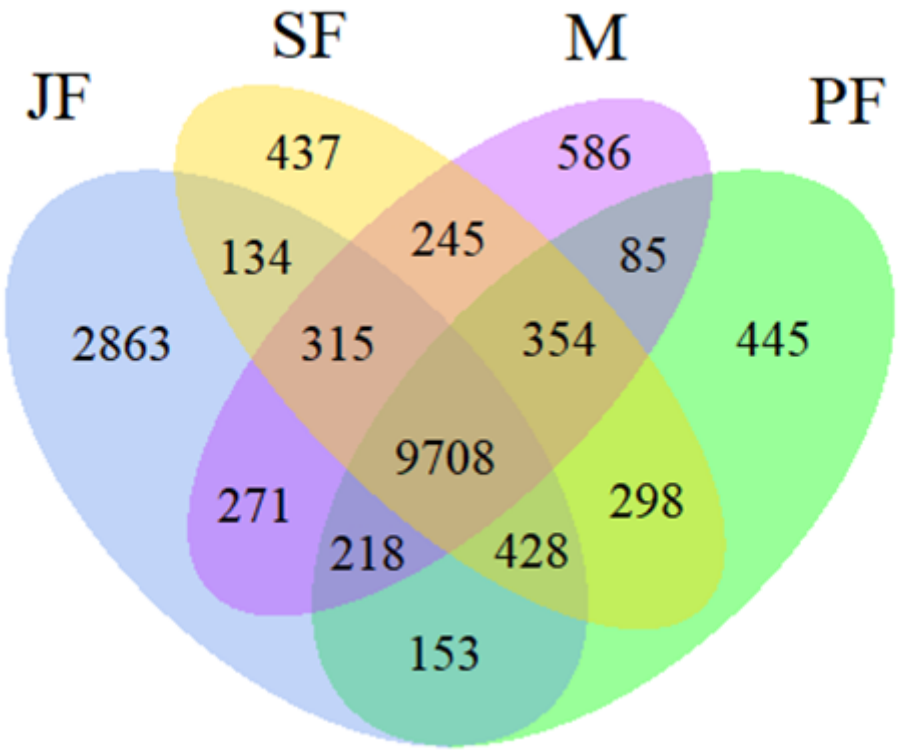

Figure 2

Venn diagram of the number of unigenes with RPKM>0.3 in four developmental stages (JF, PF, SF and M RPKM: reads per kilo bases per million mapped)

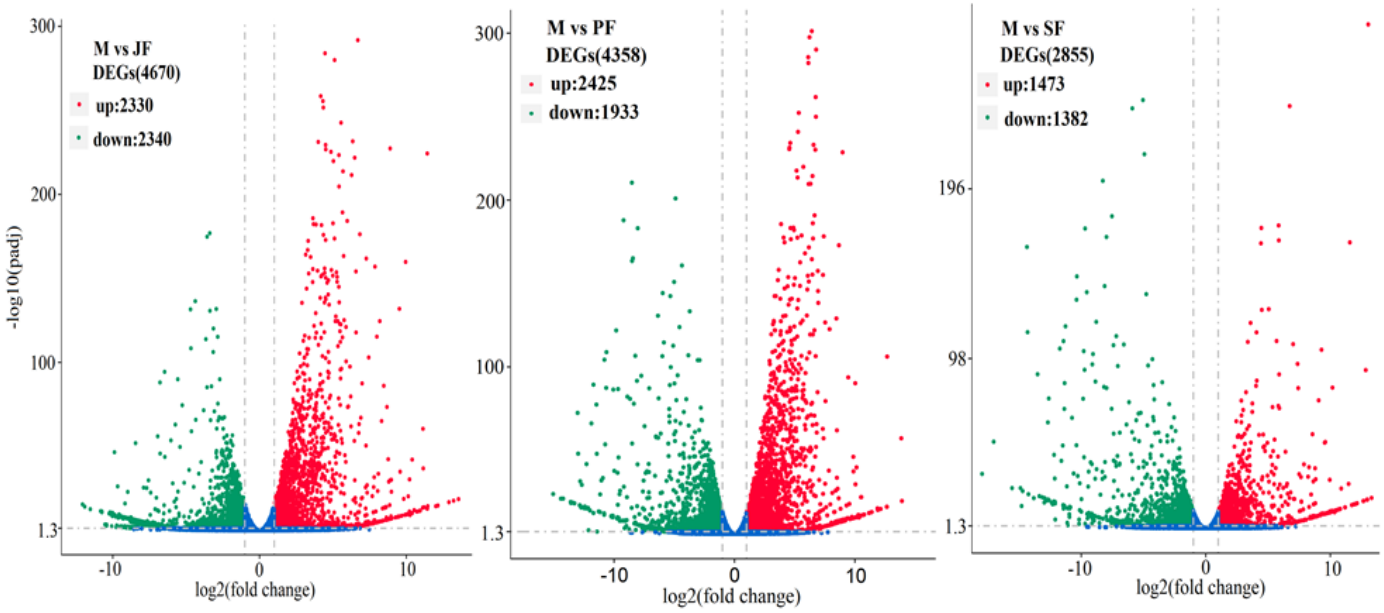

Figure 3

Volcano plot of differentially expressed genes in M vs JF, M vs PF and M vs SF

Figure 4

GO enrichment analysis of differentially expressed genes in M vs JF, M vs PF and M vs SF (red represents biological process green represents cellular component blue represents molecular function)

Figure 5

qPCR results of differentially expressed genes related to male sex determination of *D. similoides sinensis*

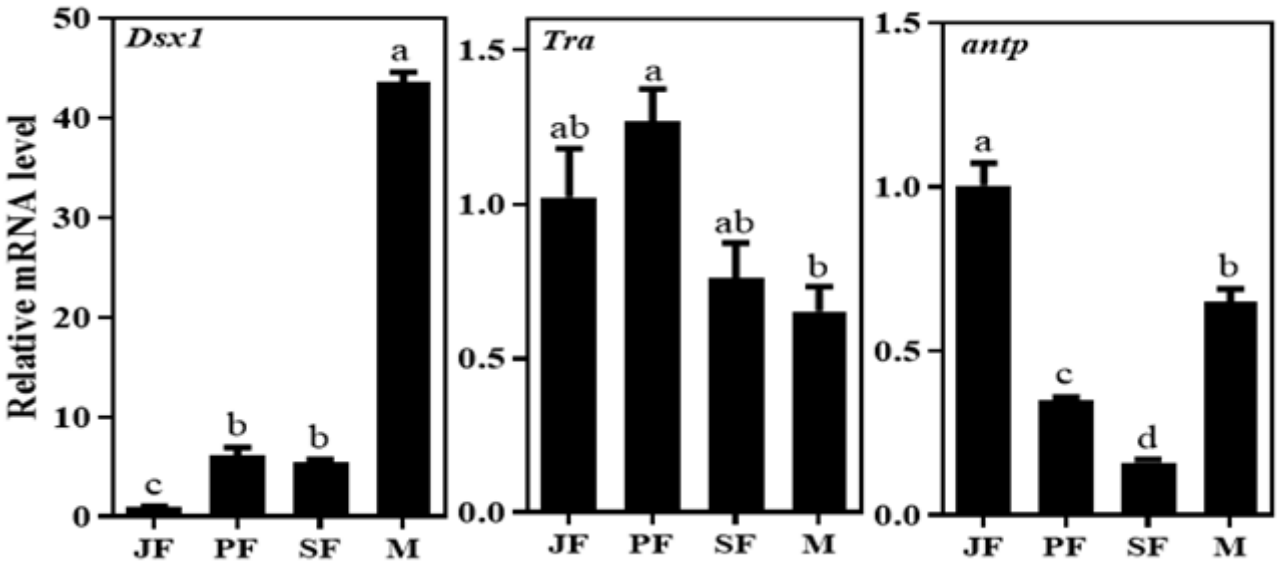

Figure 6

qPCR results of some published genes related to male sex determination in *D. similoides sinensis*

Dsx1 [13] , *antp* [12] and *Tra* [14] may be related to male sex determination in other *Daphnia* species

Supplementary Files

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