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Research paper

Analysis of the microRNA transcriptome of *Daphnia pulex* during aging

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ABSTRACT

Daphnia pulex is an important food organism that exhibits a particular mode of reproduction known as cyclical parthenogenesis (asexual) and sexual reproduction. Regulation of the aging process by microRNAs (miRNAs) is a research hotspot in miRNA studies. To investigate a possible role of miRNAs in regulating aging and senescence, we used Illumina HiSeq to sequence two miRNA libraries from 1-day-old (1d) and 25-day-old (25d) *D. pulex* specimens. In total, we obtained 11,218,097 clean reads and 28,569 unique miRNAs from 1d specimens and 11,819,106 clean reads and 44,709 unique miRNAs from 25d specimens. Bioinformatic analyses was used to identify 1335 differentially expressed miRNAs from known miRNAs, including 127 miRNAs that exhibited statistically significant differences ($P < 0.01$); 92 miRNAs were upregulated and 35 were downregulated. Quantitative real-time (qRT)-PCR experiments were performed for nine miRNAs from five samples (1d, 5d, 10d, 15d, 20d and 25d) during the aging process, and the sequencing and qRT-PCR data were found to be consistent. Ninety-four miRNAs were predicted to correspond to 2014 target genes in known miRNAs with 4032 target gene sites. Sixteen pathways changed significantly ($P < 0.05$) at different developmental stages, revealing many important principles of the miRNA regulatory aging network of *D. pulex*. Overall, the difference in miRNA expression profile during aging of *D. pulex* forms a basis for further studies aimed at understanding the role of miRNAs in regulating aging, reproductive transformation, senescence, and longevity.

1. Introduction

Small non-coding endogenous RNAs (sRNAs) mainly include microRNAs (miRNAs), piwi-interacting RNAs, and small interfering RNAs and are the regulatory molecules in living organisms. The main function of sRNAs is to regulate gene expressions related to physical growth, reproduction, aging, and diseases. miRNAs (about 22 nt) are the dominant factors of RNA silencing and were first identified in *Caenorhabditis elegans* in 1993 (Lee et al., 1993; Wightman et al., 1993). miRNAs bind to the 3′-untranslated regions (3′-UTRs) of target protein-coding mRNAs in the "seed region" and block the initiation or elongation step of transcription (Bartel, 2009). In addition, miRNAs can respond to various environmental stresses (Carnavale et al., 2013; Chen et al., 2015). Recently, scientists have performed extensive research on the effects of miRNAs on animals such as *C. elegans* (Grad et al., 2003), mouse (Tang et al., 2012), *Danio rerio* (Thatcher et al., 2007), and *Apostichopus japonicus* (Lu et al., 2014). To date, 28,645 hairpin precursor miRNAs that express 35,828 mature miRNA products have been identified in 223 species and submitted to the miRBase database (http://www.mirbase.org/).

Daphnia pulex, also known as the "water flea," belongs to the phylum Arthropoda, subphylum Crustacea, and order Cladocera, and it is an important freshwater zooplankton. Most species that belong to Cladocera exhibit a particular phenomenon that regulates the mode of reproduction from parthenogenesis (asexual) to sexual reproduction during environmental deterioration (Kleiven and Hobæk, 1992; Cao et al., 2001). Species that belong to the genus *Daphnia* are certainly good specimens for research on reproductive transformation and aging because they have characteristics such as strong agamogenesis ability, rapid growth, and simple tissue structures, and they exhibit significant changes during different periods of development (Jiang and Du, 1979; Shaw et al., 2008). These characteristics are crucial for the perpetuation of the species. Scientists have conducted extensive research on the growth- and sex-related genes found in cladocerans. Genes of the sex-

Abbreviations: miRNA, microRNA; sRNA, smallRNA; (qRT)-PCR, quantitative real-time PCR; DE miRNA, different expression microRNA; HSP70, 70-kDa heat shock protein; NGS, next-generation sequencing

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determining pathway, such as *Doublesex* (*Dsx*), *Transformer* (*Tra*), and *Chk1*, have been investigated in different reproductive models (ephippial female, parthenogenetic female, and male) of *D. pulex* and *Daphnia magna* (Kato et al., 2010; Kato et al., 2011; Zhou et al., 2013; Chen et al., 2014b; Guo et al., 2015). During the culture of *Daphnia*, the frequency of ephippial females and males increases obviously with an increase in the number of days. A previous study has shown that the expression level of the *senescence-associated protein* (*SAP*) gene is higher in ephippial females than in parthenogenetic females (Liu et al., 2014b). These studies may hint about the close connection between cyclical parthenogenesis mechanisms and the aging process in *D. pulex*. The 70-kDa heat shock protein (HSP70) and 90-kDa HSP (HSP90) have been proven to regulate the life span of *Daphnia* species (Schumpert et al., 2014; Chen et al., 2014a, 2014b). However, we still need to improve our understanding of biological development and molecular regulatory mechanisms underlying reproductive transformation during the aging process in *D. pulex*.

Fortunately, rapid progress in next-generation sequencing (NGS) technologies has provided an unprecedented sensitivity for characterizing the sRNA transcriptome in complex biological samples for identifying the potential functions and target genes of sRNAs. Recently, sRNA transcriptomes of many species, such as *Bombyx mori* (Wu et al., 2013), *Blattella germanica* (Cristino et al., 2011), *Pelteobagrus vachelli* (Zhang et al., 2016), *Ovis aries* (Zhang et al., 2013), and *Spiroplasma eriocheiris* (Ou et al., 2012), have been identified using NGS technologies. There is a lot of interest in the sRNA transcriptome of *Daphnia* species. The complete sequence of the genomic DNA of *D. pulex* has been described, and the results show that the size of the genome is about 199 Mb (Cristescu et al., 2006; Colbourne et al., 2011). Genomic analyses of the *D. pulex* peptidome have also been completed (Christie et al., 2011). Liu et al. (2013) used a bioinformatic approach to predict 252 pre-miRNAs that may produce 262 functional miRNAs in *D. pulex*. sRNA sequencing of *D. magna* for epigenetic regulation has been completed using the Illumina HiSeq 2000 NGS platform (Ünlü et al., 2015). Chen et al. screened differentially expressed microRNAs (DE miRNA) of *D. pulex* under Cd stress; these miRNAs might increase Cd tolerance by suppressing cellular growth and proliferation by GTPase and cuticle protein pathways, which switch cellular energy allocation to detoxification processes (Chen et al., 2016). However, there is limited information on the sRNAs for aging in cladocerans. HiSeq sequencing and bioinformatics have been widely used to identify and analyze the functions of aging-related miRNAs in *D. pulex*. We attempted to identify aging-related miRNAs by comparing the expression profiles and potential functions of the miRNAs at two development stages of *D. pulex*. We aimed to identify the role of miRNAs in controlling the aging process in *D. pulex* and understand the relationship between age and cyclical parthenogenesis in Cladocera.

2. Materials and methods

2.1. Sample collection and total RNA isolation

Daphnia pulex specimens were collected from a lake in Minhang District (Shanghai, China) and maintained for > 6 years in our laboratory; 100 vital individuals (body length, 3.0 ± 0.3 mm) were cultured in a 1-L glass jar at 25 ± 1.0 °C and pH 7.0–7.5 in an incubator (photoperiod, 14 L:10D). "Banta muck nutrient solution" (1.5 g rabbit dung, 2 g dry straw, and 20 g soil per liter of water) was boiled and cooled, and the filtered liquid was used for culture (Jiang and Du, 1979). *Chlorella vulgaris* (4.0 × 106 cells/mL) was added on alternate days as a food source. The newborns were separated for culture in a new jar.

One-day-old (1d), 5-day-old (5d), 10-day-old (10d), 15-day-old (15d), 20-day-old (20d), and 25-day-old (25d) *D. pulex* specimens were cultured in ddH_2O for 5 h. Total RNA was extracted using the TransZol reagent (TransGen Biotech, Beijing, China), according to the manufacturer's instructions. The integrity of the extracted RNA was tested using 1% agarose gel electrophoresis. The extracted RNA was stored at −80 °C till further use.

2.2. sRNA library construction and sequencing

The extracted RNAs (1d and 25d) were submit to Hangzhou 1gene Technology Corporation (China) for quantitation, and sRNA library preparation and sequencing were performed using the Illumina HiSeq 2000 NGS platform.

2.3. Bioinformatic analysis of the sequencing data

To obtain clean sRNA reads, the raw data were analyzed. To further sort and annotate the clean sequences, the repeat sequences and candidate rRNAs, scRNAs, snoRNAs, snRNAs, and tRNAs were identified using (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker), GenBank (https://www.ncbi.nlm.nih.gov/genbank/), Rfam11.0 (http://rfam.xfam.org/), and miRBase databases. sRNAs belonging to intron or exon sequences were used to identify the fragments formed by mRNA degradation. To make annotate only unique sRNAs, we arranged the information on the basis of priority (rRNA and others > known miRNA > repeat > exon > intron). sRNAs without any annotation were expressed as "unann."

miRNAs are usually processed by Dicer from pre-RNA; thus, restriction site specificity causes the first site of a mature miRNA sequence to show preference for U, and the tenth site prefers A because it is the splice site of the miRNA while splicing the target gene. Further characterization of known miRNAs was performed using the miRBase database.

2.4. Prediction of novel miRNAs

Prediction of the candidate miRNAs was primarily performed using the biometrics of the miRNAs. Transcription start sites of the miRNAs were located in the gene intergenic spacers, introns, and inverted repeats of the coding sequences. The pre-miRNAs with a hairpin structure were cut using Dicer enzymes to produce mature miRNAs. The minimum free folding energy for the miRNA precursor sequence should be < −18 kcal/mol. Mireap (https://sourceforge.net/projects/mireap/) was used to predict novel miRNAs.

2.5. Prediction of miRNA targets and their functional annotation

To understand the functions of the miRNAs, target genes of different expression miRNAs need to be predicted. Target genes were screened from the *D. pulex* transcriptome data (undisclosed) and used to obtain the 3′-UTR sequences. The target genes of DE miRNAs were predicted using miRBase, miRanda (http://www.microrna.org/microrna/home.do), and TargetScan (http://www.targetscan.org/vert_71/).

For the classification and enrichment analyses, DE miRNAs were analyzed using Gene Ontology (GO, http://geneontology.org/) for functional annotation. The genes were classified into molecular function, biological process, and cellular component categories. Then, the KEGG database (http://www.kegg.jp/kegg/pathway.html) was used for the pathway analysis of the target genes.

2.6. Expression analysis of D. pulex miRNAs by using qRT-PCR

Nine significant miRNAs were analyzed using qRT-PCR. The primers for these miRNAs have been listed in Table 1. U6 was used as the internal control. The analysis was performed using LightCycler 480 (Roche, Germany), and the reaction conditions were as follows: 30 s at 94 °C (first segment, one cycle), 5 s at 94 °C, 34 s at 60 °C, 34 s at 72 °C (second segment, 45 cycles), and the dissociation stage (third segment, one cycle) for the dissociation curve. Melting curves were analyzed after amplification. All reactions were run in triplicate. The relative

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