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Comparative transcriptome preliminary reveals the molecular mechanism of the growth rate of *Procambarus clarkii*

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ABSTRACT

The red swamp crayfish (*Procambarus clarkii*) is a well-known invasive species. It has become one of the most important freshwater aquaculture resources in China. In this study, we reported the karyotype and whole mitochondrial genome of *Procambarus clarkii*. In addition, we performed transcriptome analysis of *Procambarus clarkii* from two different body size populations. The three large individuals, namely, the LS group, were randomly chosen, with body lengths and weights of 16.24–18.85 cm and 38.96–45.36 g, respectively. The three small individuals, namely, the SS group, were randomly chosen, with body lengths and weights of 10.50–12.70 cm and 19.56–25.95 g, respectively. Comparative transcriptomic analysis of *Procambarus clarkii* characterized 36 differentially expressed genes (DEGs), comprising 20 upregulated genes and 16 downregulated genes in the SS group. Functional analyses revealed that four genes (*metalloreductase STEAP4-like* (*Steap4*), *myosin heavy chain fast skeletal muscle*, *muscle-like* (*MH*), *vitelline membrane outer layer protein I-like protein* (*VMO-I*), *and secreted protein acidic and rich in cysteine* (*SPARC*)) were involved in the regulation of cell proliferation, muscle growth and energy metabolism, suggesting that four genes were related to growth in *P. clarkii*. We validated the expression levels of these four differentially expressed genes in LS and SS using Quantitative real-time PCR. These results provide insights into the regulatory mechanisms underlying the difference in *P. clarkii* growth.

1. Introduction

Procambarus clarkii, often known as red swamp crayfish, is a freshwater crayfish native to South–Central America and northeastern Mexico [1]. *Procambarus clarkii* has become widely distributed throughout the world and is currently found in Europe, Africa, Central and South America, and Asia [2–4]. This species has a better adaptive capacity and is often tolerated to a variety of environmental conditions due to its excellent immune system and high growth rate [5]. In China, red swamp crayfish are cultured as an economically important freshwater species and have become a source of food due to their richness in protein, vitamin B and minerals [6].

In recent years, breeders have focused on the growth rate and body size of *P. clarkii* for commercial benefits. However, research on its growth rate has not yet been reported. At Dongting Lake in China, we found two populations of *P. clarkii* with different body sizes. We collected them and homogenized cultured them at Hunan Normal University. We found that their different body sizes did not result from heterozygous environmental conditions. In this study, we are concerned with the molecular mechanisms of the growth rate of red swamp crayfish. Transcriptome sequencing has been widely used in the genetic breeding of shrimp and crabs [7]. This technology has provided an efficient approach to sequence full-length cDNA molecules and has been successfully applied to whole-transcriptome profiling in some species, including *Litopenaeus vannamei* [8], *Macrobrachium rosenbergii* [9] and *Oratosquilla oratoria* [10]. In addition, comparative transcriptome analysis has been utilized in both plants and animals to characterize the potential key genes for domesticating traits [11,12]. We sequenced and analyzed the transcriptome of liver tissues of red swamp crayfish of different body sizes.

Chromosomes are the carriers of genetic material, and karyotype analysis is the basis of cytogenetics. Mitochondrial DNA (mtDNA), as a genetic system outside the nucleus, has attracted much attention for integrity, versatility, autonomy, and miniaturization. MtDNA plays an important role in the evolutionary classification and identification of species. It is often used to reconstruct the phylogenetic tree of aquatic animals [13–15]. In this study, we reported the chromosome number and

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mtDNA of *Procambarus clarkii*. We used chromosomes and mtDNA to characterize the basic characteristics of *P. clarkii* and obtain a basic understanding of what we know about *P. clarkii*. These studies will provide basic data for genetic improvement and breeding of new varieties of *P. clarkii*.

2. Materials and methods

2.1. Ethics statement

Procambarus clarkii were certified under a professional training course for laboratory animal practitioners held by the Institute of Experimental Animals, Hunan Province, China (Certificate No. 4263). All *P. clarkii* were euthanized using 2-phenoxyethanol (Sigma, United States) before dissection. This study was carried out in accordance with the recommendations of the Administration of Affairs Concerning Experimental Animals for the Science and Technology Bureau of China. The protocol was approved by the Administration of Affairs Concerning Experimental Animals for the Sciences and Technology Bureau of China.

2.2. Sample collection and preparation

Two population *P. clarkii* groups (LS and SS) were obtained from Dongting Lake in Yiyang city, Hunan Province, China. The *P. clarkii* were reared in water tanks at 20–23 °C and fed twice daily with a commercial diet. The water was changed twice a week.

2.3. Preparation of chromosome

Chromosome preparation followed the method provided by Segura et al. [16]. In brief, the testes of *P. clarkii* individuals were placed in a petri dish containing 0.8% NaCl solution. The samples were cut into pieces, moved into tubes and flushed with a pipette. After 30 min, the cell suspension was removed and centrifuged at 1,000 rpm for 5 min. The supernatant was discarded, and 0.075 mol/L KCl was added to the pellet. The mixture was stirred by flushing with a pipette repetitively. The mixture was then centrifuged again, and Carnoy's fixative (methanol and glacial acetic acid, 3:1) was added for 15 min. After three washes with Carnoy's fixative, 1 mL of cell suspension was dropped onto clean slides and dried by flame. The dried preparation was stained with 2% Giemsa's stain. The best metaphase arrays from the slides were selected and microphotographed.

2.4. Analysis of mitochondrial DNA sequencing

Fresh liver tissue was isolated from starved *P. clarkii* to obtain mtDNA. MtDNA was extracted by using a modified protocol and used to construct short-insert libraries with a size of 350 bp for sequencing on the Illumina NovaSeq platform under the PE 150 bp protocol. The mtDNA was assembled by using the program the de novo assembler SPAdes 3.11.0 [17].

2.5. Measurement of the size of Procambarus clarkii

Two different size populations (LS group and SS group) of *P. clarkii* were obtained according to the different sizes. Each group of 20 individuals was randomly selected. The data was shown in Table 1. The software SPSS 19.0 [18] was used to analyze the difference between the two groups.

Table 1
Comparison of body length and body weight in the LS group and SS group.

	Body Length	Body Weight
LS group	16.48 ± 0.24	43.23 ± 2.50
SS group	11.11 ± 0.50	25.82 ± 2.01

2.6. RNA sequencing library construction and Illumina sequencing

For the investigation of *P. clarkii* growth, we collected liver tissues from both larger-sized *P. clarkii* (LS) and small-sized *P. clarkii* (SS). We performed three independent collections from different individuals for replication (accession numbers of three individuals from the LS group and three from the SS group were SRR15461231, SRR15498210, SRR15496475, SRR15509780, SRR15508059, and SRR15522880). For each sample, we isolated and purified total RNA using a *Trans*-up RNA isolation kit (Biostar, Shanghai, China) according to the manufacturer's protocol. The yield and purity of each RNA sample were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the integrity of all RNA samples was assessed by 1% agarose gel electrophoresis. Total RNA was treated with DNase to remove DNA contamination.

High-quality ribonucleic acids were constructed into cDNA libraries. We separated poly(A) by oligo-dT beads (Qiagen, Dusseldorf, Germany). All mRNAs were divided into short segments by adding fragmentation buffer. The first strand of cDNA was synthesized by four polymer random primer. Then, the second cDNA strand was synthesized by DNA polymerase I and endonuclease. The cDNA fragment was purified using a quick PCR extraction kit. To carry out terminal repair poly(A) addition, we rinsed the purified cDNA fragments with EB buffer and then connected them with the ligated barcodes. Constructing the final cDNA library required separated fragments of suitable size for sequencing standards from the gel and enrichment by PCR amplification. BioMarker Technologies (Beijing, China) sequenced the cDNA library on the Illumina sequencing platform (Illumina HiSeq 2500) using paired-end technology in a single run. The images and basic calls were made by using an illumina GA processing pipeline.

2.7. De novo assembly and functional annotation

Prior to assembly, we removed PCR duplications, adapter sequences and low-quality sequences with <90% identified nucleotides from raw reads using the QC_pe pipeline [29]. Then, the clean reads of high quality from all samples were merged together and assembled using the Trinity package [30] to construct unique consensus sequences as the reference sequences. Then, we used the longest isoform from each TRINITY assembly to generate an unigene by using the Trinity2Unigene.pl script from Feng et al. [19] and further reduced the redundancy of unigenes using CD-HIT-EST v.4.7 (with the parameter -c 0.95) [31]. We identified the coding regions (cds and protein sequences) by using TRANSDECODER [32].

We identified homologs of assembled transcripts by using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) [20] to search against the NCBI protein "nr" database at an e-value of 1e−5. Gene ontology (GO) terms were determined by using the BLAST2GO program [21]. KEGG annotations were assigned with Blast2GO KEGG mapping [22].

2.8. Identification of differentially expressed genes

Sequencing reads were remapped to the reference transcriptome by the align_and_estimate_abundance.pl program from TRINITY. For each gene, the expression level was measured by FPKM (fragments per kilobase exon model per million mapped fragments) for each sample. Differentially expressed genes were determined with DESeq2 [33]. A default dispersion value of 0.1 was used to calculate the differential expression for samples with no replicates. Transcripts exhibiting ≥ 4-fold change (Log2FC) in expression and <0.001 false discovery rate (FDR) were considered significant DEGs.

2.9. Validation of RNA-seq results by qPCR

Quantitative real-time PCR was performed on four selected genes

(*Steap4*, *MH*, *VMO-I*, *SPARC*). Within each of the categories for upregulation and downregulation in liver tissue, we selected four genes for quantitative real-time PCR (qPCR) validation, and specific primers (Supplementary Table 1) were designed using the Primer Premier 5.0 program [23]. The total RNA of the liver was extracted from 6 individuals which included three large individuals and three small individuals. Q-PCR was performed using the SYBR premix ExTaq Kit (TaKaRa, Dalian, China). The thermal profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. The mRNA expression of each gene was quantified relative to β-actin (forward primer, 5′-TCTTGGACTCTGGTGATG-3'; reverse primer, 5′- GTAGTTGTGAA GGTGTAGC-3′). The average cycle thresholds (CT) used to determine fold-change. The relative quantification of gene expression was reported as a relative quantity to the control value, and all experiments were performed with three biological replications. The relative expression levels were calculated using the equation $(2)^{-\Delta\Delta CT}$ [24].

3. Results

3.1. Comparison of body length and body weight

The means and standard deviations (SD) for body length and body weight were given in Table 1. The body length and body weight were significantly different between the LS group and SS group ($P < 0.05$).

3.2. Chromosome number and karyotype analysis

The divalent chromosomes of spermatocytes in metaphase meiosis were short and rod-shaped (Fig. 1 a). The number and frequency of chromosomes were counted. For the number of divalent chromosomes in primary spermatocytes, 58% of cells had a chromosome number of N = 94. These results suggested that the diploid chromosome number was 2 N = 188, and the haploid number was N = 94 in *P. clarkii*.

According to the position of the centromere, the meiotic phases of *P. clarkii* were divided into three groups: group A (No. 1-55) (Fig. 1 b), in which it was possible to clearly identify the position of the centromere as a centromere divalent; group B (No. 56-76) (Fig. 1b), with a total of 22 subcentral or subterminal centromeric bivalents; and group C (No. 77-94) (Fig. 1b), where the centromere was not found and the bivalents appeared as small dots and were classified as end centromere bivalents. Therefore, the karyotype formula was N = 55 M + 22 (SM, ST) + 17T (Fig. 1b).

3.3. Analysis of mtDNA

The mtDNA of *P. clarkii* (GenBank No: MZ595228) was 15,936 bp in length. It consisted of 13 protein-coding genes, 22 transfer RNA genes, 2 rRNA genes, and 1 control region (D-loop). Except 2 protein-coding genes (*Cob* and *Nad6*) and 7 tRNA genes ($tRNASer^{TGA}$, $tRNA^{Thr}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Gln}$, $tranSer^{TCT}$, and $tRNA^{Asn}$) encoded on the L-strand, all other genes were encoded on the H-strand.

The common start codon ATG was found in nearly all protein-coding genes. Five genes (*cob*, *nad6*, *nad2*, *cox1*, and *nad3*) used ATT as start codons. In addition, the start codon of *nad1* was ATA. Three types of stop codons were used for the coding genes: TAA for *cob*, *nad6*, *nad4L*, *nad4*, *nad5*, *nad2*, *cox1*, *atp8* and *cox3*; TAG for *nd3* and *nad1*; and an incomplete stop codon T-for *COII*. The total length of mtDNA protein-coding genes in *P. clarkii* was 11,129 bp (Fig. 2).

3.4. Sequencing, de novo assembly and functional annotation

We assembled transcripts from three large and three small *P. clarkii* individuals. We got a total of 58,897 unigenes. In these unigenes, there were 690 unigenes (1.17%) 200-300 bp in length, 36,861 unigenes (62.59%) 300-500 bp in length, 11,936 unigenes (20.27%) 500-1000 bp in length, 5,842 unigenes (9.92%) 1000-2000 bp in length, and 3,569 unigenes (6.06%) more than 2,000 bp in length. The total length was 42,654,192 bp, and the N50 length was 957 bp with an average length of 724.22 bp. In addition, we assembled transcriptomes for large- and small-*sized P. clarkii* separately (Supplementary Table 2). Both large- and small-sized *P. clarkii* had more unigenes than the reference transcripts. More unigenes were discovered in small *P. clarkii* than in large *P. clarkii*.

We totally annotated 32,780 CDS. In these CDSs, 18,376 were annotated to the GO database (Fig. 3), 22,633 to the KEGG database, 15,468 to the KOG database, 19,857 to the Pfam database, 19,934 to the Swiss-Prot database, and 29,934 to the NR database. We found a total of 36 genes differentially expressed between large- and small-sized *Procambarus clarkii* ($P \leq 0.01$). Compared with large *Procambarus clarkii*, there were 20 upregulated genes and 16 downregulated genes in small *Procambarus clarkii*. We found four genes in these differentially expressed genes (DEGs), and we supposed that these genes resulted in different body sizes between these two populations (Table 2, Supplementary Table 3, Fig. 3).

3.5. Analysis of functional enrichment

Gene ontology (GO) terms were used to classify the function of the predicted *P. clarkii* transcriptome. For the 36 DEGs, we conducted an analysis of GO enrichment analysis. We found that these genes were enriched in GO terms, including oxidoreductase activity, homeostatic process and regulation of biological quality (Supplementary Fig. 1).

3.6. Validation of differentially expressed genes by qPCR

Quantitative real-time PCR (qPCR) was performed on four selected genes (*Steap4*, *MH*, *VMO-I*, *SPARC*). The expression patterns of the four

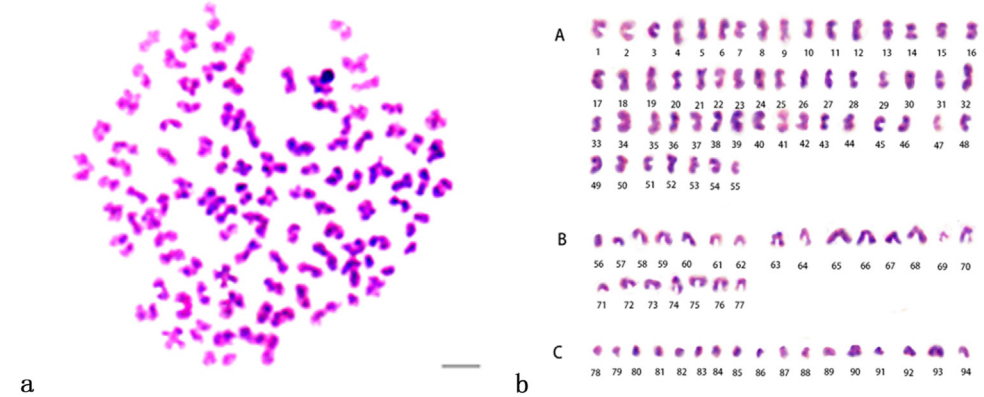

Fig. 1. (a) Meiosis Metaphase chromosomes of spermatogonia cells of *Procambarus clarkii*. (b) Karyotype analysis of *Procambarus clarkii*.

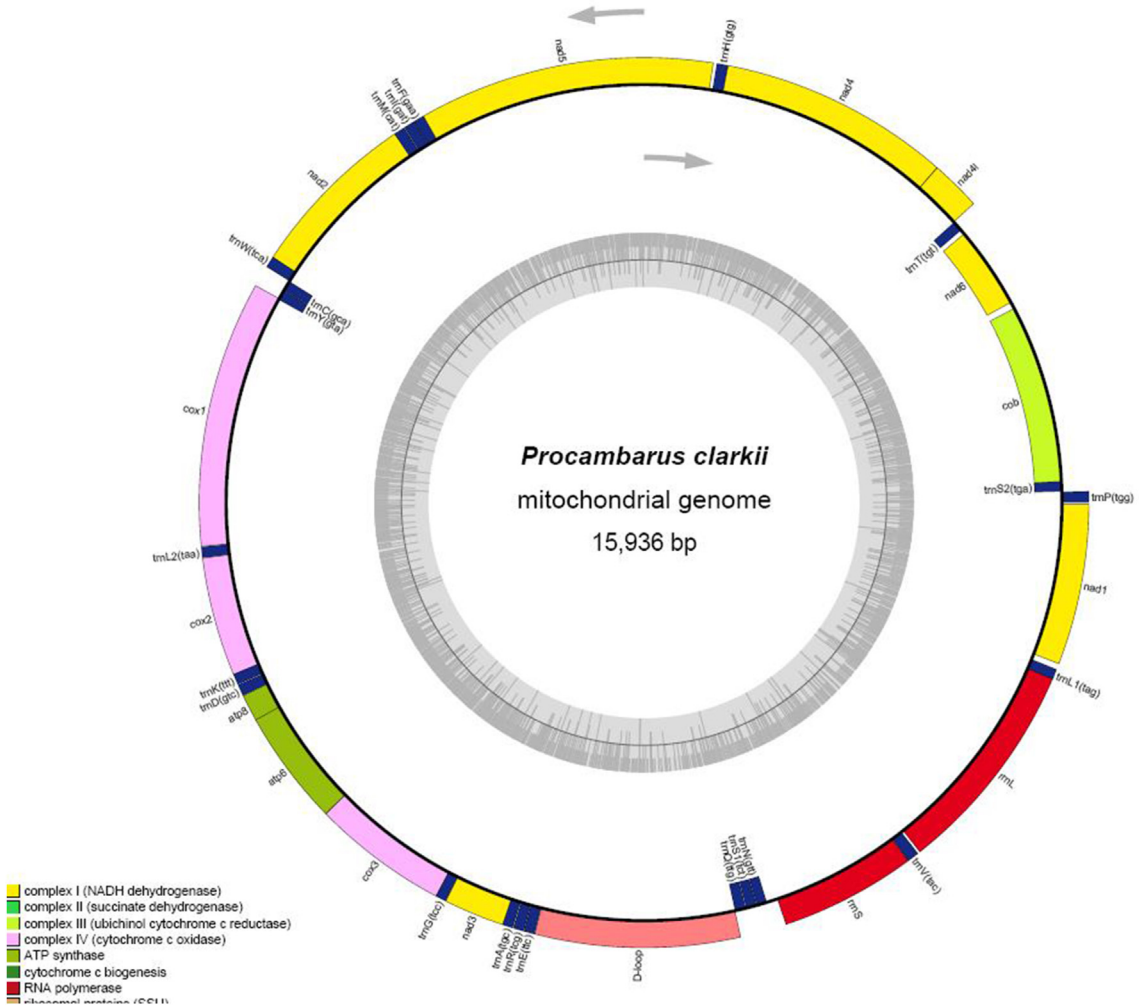

Fig. 2. Mitochondrial genome structure of the prawn *Procambarus clarkii.*

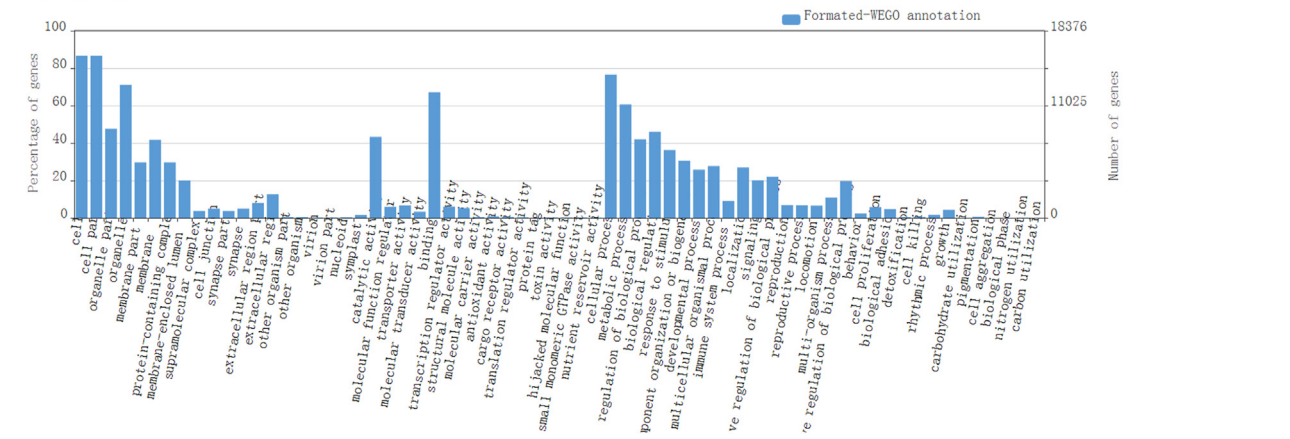

Fig. 3. Analysis of Go enrichment analysis.

Table 2
Summary of the four DEGs related to growth.

Gene id	Gene name	log2FoldChange	FDR*
DN763_c0_g1	metalloreductase STEAP4-like (*Steap4*)	2.879352006	0.011222
DN1780_c0_g1	myosin heavy chain, muscle-like (*MH*)	−1.478080998	0.049746
DN7821_c0_g1	vitelline membrane outer layer protein I-like protein (*VMO-I*)	−2.609961912	0.026795
DN27057_c0_g1	Secreted protein acidic and rich in cysteine (*SPARC*)	−1.315385112	0.044816

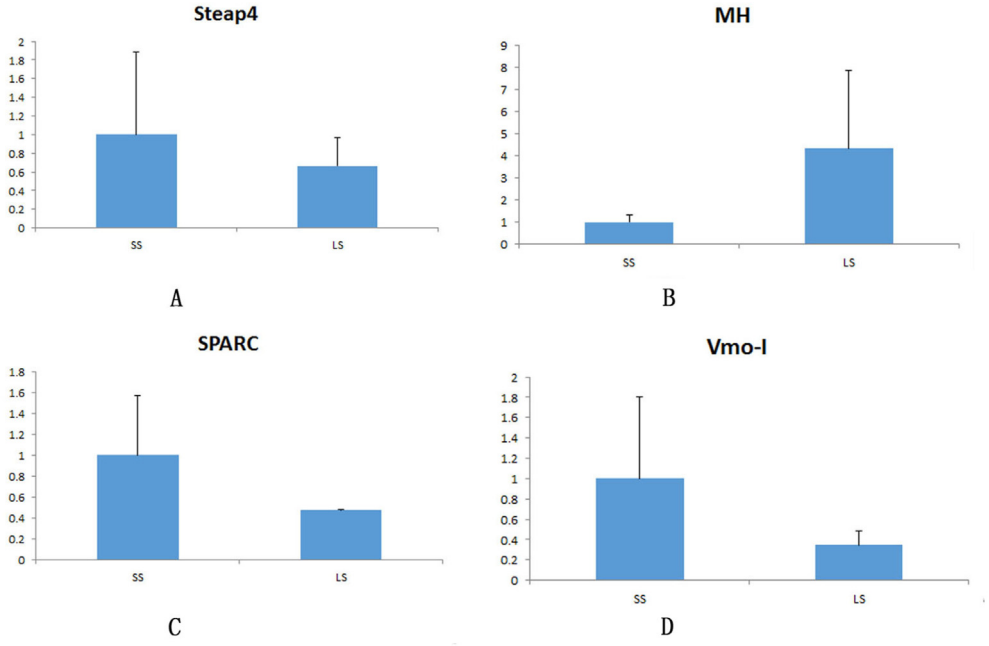

Fig. 4. Real-time PCR analysis of the four DEGs (A) *Steap4*, metalloreductase STEAP4-like; (B) *MH*, myosin heavy chain fast skeletal muscle, muscle-like; (C) *SPARC*, secreted protein acidic and rich in cysteine; (D) *Vmo-I*, vitelline membrane outer layer protein I-like protein. In each panel, LS means large individuals and SS means small individuals.

genes by qPCR ranged from significantly different to similar to those indicated by the RNA-seq analysis (Fig. 4).

4. Discussion

Research on aquatic animal domestication has been carried out for several years [25,26]. *Procambarus clarkii* is one of the most well-known invasive species in the world [1]. It has been observed in China for more than one hundred years, and it has now been observed in almost all forms of freshwater in most Chinese provinces [27]. It has also been domesticated as a popular food resource for nutritional meat with a high protein content. The meat of *P. clarkii* is rich in many essential mineral elements, such as magnesium, which is beneficial for the regulation of heart activity. Regular use of crayfish meat can maintain nerve and muscle excitability. In this study, we investigated the chromosome number and mtDNA of red swamp crayfish. In addition, we performed comparative transcriptome analysis between large- and small-body-sized populations.

In the 94 chromosomes of *P. clarkii*, most of these chromosomes are spotty in shape. It is a distinctive feature of crustacean chromosomes. By analyzing its karyotype, the formula was N = 55 M + 22 (SM, ST) + 17 T. There were no terminal chromosomes. In addition, we found that it is difficult to distinguish the subtelomeric from the submodel centromeres.

According to the characteristics of chromosome number, morphology and centromere position of *P. clarkii*, we can determine the population type of *P. clarkii*, which is of great significance to understand the evolution of *P. clarkii* population. The study on the mtDNA structure of *P. clarkii* is of great significance to the origin and differentiation of this species. For the *P. clarkii* mtDNA, the length is similar to most fishes. The mtDNA has a typical annular structure. It consists of 13 protein-coding genes, 22 transfer RNA genes, 2 rRNA genes, and 1 control region (D-loop). Through the analysis, it is found that the mtDNA of crustaceans and fish are similar, indicating that there are similarities between fish and crustaceans in mtDNA. Knowledge of the mechanisms governing growth processes at the molecular level is crucial and could be directly applied to the crawfish industry [28]. In the DEGs between large- and small-sized populations of red swamp crayfish, we found many genes of interest. Myosin is a diverse superfamily protein and plays a molecular role in a number of biological motilities. Generally, muscles are classified into ordinary and dark muscles, which correspond to fast and slow skeletal muscles. Myosin heavy chain fast skeletal muscle, muscle-like (*MH)* proteins which is composed of two myosin heavy chains (MHCs) each with approximately 200 kDa and four light chain subunits (MLC) each with approximately 20 kDa, where each MHC binds two MLCs [29]. In adult specimens of black tiger *Penaeus monodon* and Pacific white *Penaeus vannamei* shrimp, the *MH* protein is the major protein in skeletal muscle [30]. Two genes encoding myosin heavy chain (MHC), a large subunit of the myosin molecule, were cloned from abdominal fast skeletal muscle defined as MHCa and MHCb according to our previous study on *Marsupenaeus japonicas* [31]. *MH* could promote the growth of muscle. On the one hand, we found that the gene expression of *MH* in LS was more abundant than that in SS. On the other hand, the *MH* gene could affect the growth of *P. clarkii*. We suppose that this gene could be an important target or molecular marker in red swamp crayfish breeding. Metalloreductase STEAP4-like (*Steap4*) is abundantly expressed in human adipose tissue, *steap4* localizes to the plasma membrane of adipocytes, and *steap4* expression is induced by TNF-α in adipose tissue. *Steap4* is associated with obesity and insulin resistance. The expression of *steap4* in adipocytes is normally induced by nutritional stress, leptin, and proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β, and interleukin-6, where it promotes insulin sensitivity [32]. In humans, the downregulated expression of *steap4* could cause body weight changes compared with the controls [33]. In this study, we found that the expression level of *steap4* in large *Procambarus clarkii* was higher than that in small *procambarus clarkii*. Through the dissection of *Procambarus clarkii*, the fat thickness of large *Procambarus clarkii* was greater than that of small *Procambarus clarkii*. The result of qPCR showed that the *steap4* gene expression level of large *Procambarus clarkii* was significantly lower than that of small *Procambarus clarkii*. From the above, it can be concluded that the growth rate of crayfish is regulated by the *steap4* gene. *VOM-I* was considerably resistant to tryptic digestion. In this study, the *VMO-I* expression level of larger *Procambarus clarkii* was significantly lower than that of small *Procambarus clarkii*. We speculate that the *VOM-I* gene is closely related to the muscle tightness of *Procambarus clarkii*. *Secreted protein acidic and rich in cysteine* (*SPARC*) is a

matricellular protein with multiple functions. It regulates the assembly and organization of the extracellular matrix, modulates multiple intracellular signaling pathways and affects cell migration, proliferation and differentiation [34]. The qPCR experiment confirmed that the expression level of the *SPARC* gene in LS was significantly lower than that in SS. Therefore, the overexpression of *SPARC* may inhibit body weight gain.

In summary, our results provide a foundation for the further characterization of gene expression in LS and SS with respect to growth speed.

Author contributions

This study was conceived and designed by S.J.L.; Y.D. W contributed experimental work, most of the statistical analysis and manuscript writing; Y.D. W and S. W contributed to primer design and the bioinformatics analysis; D.S. H, H.F. T, Y.X. L and J.J.Y. contributed to the collection of experimental materials; J.L. L and D.W. W contributed to photograph collection. All authors read and approve the final manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.repbre.2021.11.003.

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