Comparative transcriptome analysis of antennae from male crayfish Procambarus clarkii during the mating period and non-mating period

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Abstract

The red swamp crayfish Procambarus clarkii is one of the global invasive species and has caused huge damage to aquaculture, biodiversity and ecology in the world. Antennal-expressed receptors are important for P. clarkii to detect olfactory cues for mate attraction, et al.. However, few olfactory and chemosensory-related genes were reported in P. clarkii until now. In the present study, we used RNA sequencing to investigate the olfactory and chemosensory-related genes of the antenna of P. clarkii during the non-mating and mating period. A total of 59218 unigenes with an average length of 1056.41 bp, and 4889 differentially expressed unigenes (DEGs) with 2128 up-regulated and 2761 down-regulated unigenes were obtained. Of which, 13 up-regulated and 9 down-regulated DEGs were identified to associated with olfaction and chemical reception, including 4 IRs or iGluRs, 8 G-protein coupled receptor, 5 transient receptor potential channels (TRP channels), 1 sodium-calcium exchanger, 1 olfactory receptor, 1 isomerase and 2 chemosensory proteins (CSPs). CSPs were preliminarily classified into pheromone receptors in male red swamp crayfish. The results of quantitative real-time reverse transcription PCR (RT-qPCR) showed that the expression trends of 8 selected unigenes were consistent, and this result also validated the RNA-Seq data. Our results provide more comprehensive data of olfactory and chemical communication mechanism after crayfish entering the mating period.

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Abstract

The red swamp crayfish, *Procambarus clarkii,* is globally an invasive species and has caused huge damage to aquaculture, biodiversity, and ecology worldwide. Antennae-expressed receptors are important for *P. clarkii* to detect olfactory and chemosensory cues for mate attraction. However, only a few olfactory and chemosensory-related genes have been reported in *P. clarkii* to date. In the present study, we performed RNA sequencing to investigate the olfactory and chemosensory-related genes of the antenna of *P. clarkii* during the non-mating and mating periods. A total of 59218 unigenes with an average length of 1056.41 bp, and 4889 differentially expressed unigenes (DEGs), among which 2128 were upregulated, while 2761 were downregulated were obtained. Further, 13 upregulated and 9 downregulated DEGs were associated with olfaction and chemical reception, including 4 IRs or iGluRs, 8 G-protein coupled receptors, 5 transient receptor potential channels (TRP channels), 1 sodium-calcium exchanger, 1 olfactory receptor, 1 isomerase, and 2 chemosensory proteins (CSPs). CSPs were preliminarily classified as pheromone receptors in male red swamp crayfish. The results of quantitative real-time reverse transcriptase PCR (RT-qPCR) showed that the trends of expression of 8 selected unigenes were consistent with RNA-Seq results. Our results provide more comprehensive data for olfactory and chemical communication mechanisms after crayfish enter the mating period.

Keywords: *Procambarus clarkii* , antenna transcriptome, RNA-Seq, olfactory, chemical communication

INTRODUCTION

Olfaction, as a major sensory stimulus, plays an important role in almost vertebrates and invertebrates. For most invertebrates, olfaction generally takes a more principal account than other sensory modalities (Krieger and Breer, 1999). Olfaction can provide essential information for crustaceans in finding food, avoiding predators, and performing other social behaviors (Breithaupt, 2011; Derby and Zimmer, 2012; Hay, 2011; Kamio and Derby, 2017; Schmidt and Mellon, 2011). Olfaction mediates the reproductive processes in crustaceans. For example, in the marine shrimp, *Lysmata wurdemanni* , the males with more olfactory sensilla search or approach the females earlier, thereby increasing their rate of mating success (Zhang et al., 2009).

Previous studies suggest that the antenna is the predominant olfactory organ of most crustaceans (Harzsch et al., 2011; Urbschat and Scholtz, 2019; Waldrop, 2013). Additionally, crustaceans receive chemical signals through several parallel channels, which can be summarized into two modes of "olfaction" and "distributed chemoreception" (Schmidt and Mellon, 2011). "Olfaction" is mediated by unimodal olfactory sensilla called "aesthetasc", which are only present on the first antenna of the crustaceans (Harzsch and Krieger, 2018). Aesthetascs are innervated by olfactory receptor neurons (ORNs) (Derby et al., 2016). "Distributed chemoreception" is mediated by various bimodal sensillae in the first antenna (Solari et al., 2017), mouthparts (Garm et al., 2004; Garm et al., 2003), chelipeds, and walking appendages (Altner et al., 1983; Schmidt and Gnatzy, 1984). It is innervated by both chemoreceptor neurons (CRNs) and mechanoreceptor neurons (MRNs). However, there is a gap in the data for olfactory and chemo-receptors of crustaceans. So far, the acknowledged olfactory-related proteins in crustaceans include ionotropic receptors (IRs), ionotropic glutamate receptors (iGluRs), G-protein coupled receptors (GPCRs), and transient receptor potential channel (TRP channel), while proteins such as gustatory receptor-like receptors (Grls) and gustatory receptors (GRs) are only found in a few crustacean species (Derby et al., 2016; Kozma et al., 2020a; Kozma et al., 2020b).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), has its origins in North-eastern Mexico and

South America (Shen et al., 2014), and was introduced to China from Japan during the 1930s (Shen et al., 2020). At present, it is an indispensable aquatic-economic animal (Liu et al., 2021). However, wild crayfish in the natural ecosystem of China have caused huge losses to agriculture, biodiversity, and aquaculture. Moreover, the red swamp crayfish is a carrier of the white spot syndrome virus (WSSV) and parasites, whereby it may cause infection and death of other commercial shrimps in case of spread without effective control (Zhu et al., 2009). Therefore, effectively and specifically controlling wild crayfish requires more attention. In recent years, control of invasive species using sex pheromone has been adopted for other species of insects (Gherardi et al., 2011; Johnson et al., 2015; Keller-Costa et al., 2014). Previous studies confirm that the males can detect sex pheromones through their olfactory organs uniaxially, and then search for the signal source or perform courtship behaviors (Oyama et al., 2020). Moreover, the male red swamp crayfish recognizes female mating receptivity by detecting the urinary components of females (Kubec et al., 2019). However, little is known about the chemosensory mechanism of action of these essential chemicals (such as sex pheromone components) in this species.

Like other crustaceans, the red swamp crayfish's antenna is the most dominating sensory organ that perceives and locates chemical signals or pheromones released by their mates (Breithaupt, 2011). Therefore, to further understand the communication mechanism through olfaction and chemosensation in male red swamp crayfish, we collected the antenna of the male crayfish in the mating period (MP) as the experimental group and the non-mating period (NMP) as the control group. Subsequently, next-generation sequencing (NGS) was performed to sequence the antenna transcriptome of red swamp crayfish in MP vs. NMP. Our findings may help better understand the mechanisms of olfactory and chemosensory responses in red swamp crayfish, and provide a solid foundation for further studies on the relationship between olfactory-related genes and semiochemicals.

MATERIALS AND METHODS

Animal collection and preparation

P. clarkii were purchased (300 in total) from Guilin city, Guangxi province, China. The average weight of the mature male crayfish was 16.22 ± 1.82 g. The length of the antenna was longer than 5 cm (Figure 1). A total of 20 crayfish were cultured in one water tank and fed twice a day on artificial food throughout the experimental period (Li et al., 2012). All experimental animals were male crayfish and had no contact with female crayfish or pheromones during the feeding process. Other conditions such as water temperature and illumination time etc., were changed naturally according to the season. We divided the crayfish into two following groups: 100 individuals were randomly selected as the experimental group and 100 as the control group. The former were fed until April (the initial stage of the MP) and the latter until January (NMP). Antennae were collected, frozen in liquid nitrogen, and stored at -80 degC until the extraction of total RNA.

Figure 1 The red swamp crayfish, *Procambarus clarkii* .

Total RNA extraction and detection

The library construction and sequencing of transcriptomes were performed by Majob Technology Co. Ltd (Shanghai, China). Total RNA extraction was performed using the Trizol reagent (Invitrogen, Shanghai, China). Both concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Invitrogen, Massachusetts, USA). The degradation of extracted RNA was detected on a 1% agarose gels and RNA integrity number (RIN) was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

Library construction and sequencing

mRNAs were enriched using magnetic beads with Oligo (dT) and randomly fragmented using fragmentation buffer. Under the action of reverse transcriptase, the fragmented mRNAs were used as templates for first-strand cDNA synthesis using random hexamer primers. Subsequently, second-strand cDNA was synthesized using DNA polymerase I and RNase H. We added an end-repair mix (including the end-repair enzyme mix and end-repair buffer) to patch the cohesive ends of the double-strand cDNA, followed by the addition of tail and sequencing adapters. Subsequently, cDNA was amplified by PCR. The cDNA library was obtained after purifying the amplification products with AMPure XP beads. Thereafter, QuantiFluor dsDNA System and Quantus Fluorometer (Promega, Madison, Wisconsin, USA) were used to detect the concentration and inter size of the library, respectively. The effective concentration of the library was accurately quantified by q-PCR (quantitative polymerase chain reaction). Finally, the processed samples were sequenced on the Illumina HiSeqXten/NovaSeq 6000 Sequencing Platform.

de novo assembly and annotation

Using SeqPrep (*https://github.com/jstjohn/SeqPrep*) and Sickle (*https://github.com/najoshi/sickle*), clean reads were obtained by filtering the sequencing adapters, primer sequences, and the low-quality sequences from raw reads. Using the Trinity software (Grabherr et al., 2011), obtained clean reads were assembled de novo into unigenes, which were the encoding sequences. The corresponding amino acid sequences were detected by TransRate (*http://hibberdlab.com/transrate/*) to obtain information on the comprehensive gene function.

The unigene sequences were compared using six databases, including the National Center for Biotechnology Information (NCBI) Non-Redundant Protein Sequence Database (Nr) (*ftp://ftp.ncbi.nlm.nih.gov/blast/db/*), Protein family (Pfam) (*http://pfam.xfam.org/*), Gene Ontology (GO) (*http://www.geneontology.org*) Swiss-Prot (*http://web.expasy.org/docs/swiss-prot_guideline.html*), Cluster of Orthologous Groups of proteins (COG) (*http://www.ncbi.nlm.nih.gov/COG/*), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (*http://www.genome.jp/kegg/*). Functional annotation was processed using the Blast2GO software (Conesa et al., 2005).

DEG enrichment analysis

The Bowtie software (Langmead et al., 2009) was used to compare clean reads with the unigene library and on combining with RSEM (Li and Dewey, 2011), the expression levels were estimated. The expression of unigenes was calculated using FPKM (fragments per kilobase per million) (Mortazavi et al., 2008) between two libraries. Then, the DEGs were analyzed using the DESeq2 software (Varet et al., 2016). The resulting P -values were adjusted to control the false discovery rate (FDR). The DEGs were filtered using the threshold of FDR < 0.01 and $|$ log2 (Fold Change) $|[?]$ 1. Subsequently, GO and KEGG pathway enrichment analyses were conducted using the Goatools software (Klopfenstein et al., 2018).

Real-time qPCR (RT-qPCR)

To verify the results of our sequencing analyses, we selected 8 olfactory-related genes from the male antennae of red swamp crayfish for RT-qPCR analysis. The total RNA was reversely transcribed into first-strand cDNA using the PrimeScript TM 1st stand cDNA Synthesis Kit (TaKaRa, Shanghai, China), following which the newly synthesized cDNA was used as the template for RT-qPCR. Specific primers were designed using the Primer 5.0 software. The β-actin gene was considered an internal normalization control. The primer

sequences of 8 DEGs used for RT-qPCR are listed in Table S1. The RT-qPCR reactions (20 μl) contained 2 μl cDNA, 1 μl of each primer, 1 μl dNTP Mix, 10 μl 2×SYBR real-time PCR premixture, and 6 μl RNase free sterilized ultrapure water. The reaction procedure was as follows: 95 °C for 5 min, followed by 40 cycles with 95 °C for 15 s, and 60 °C for 30 s. To confirm reproducibility, the RT-qPCR reaction for each sample was performed in three technical replicates and three biological replicates. The level of expression of selected genes was calculated using the $2^{-\Delta\Delta\hat{}\tau}$ method (Livak and Schmittgen, 2001). Comparative analyses for each target gene among different samples were analyzed using independent sample$t$ -tests (SPSS, version 25.0).

RESULTS

Assembly and splicing

A total of 133,462,976 and 125017008 raw reads were obtained from the NMP and MP groups, respectively. After quality control, we obtained at least 5.98 Gb reads per sample. A total of 130343776 clean reads were obtained from the NMP group and 122633780 from the MP group with a Q30 score > 92.04 %, and the G + C content ranged from 47.83% - 49.05% in NMP group and 44.38 %- 47.45% in MP group (Table S2). Moreover, clean reads were randomly assembled into 78138 transcripts with an average length of 1147.85 bp and an N50 length of 2379 bp. The clean reads were further assembled into 59218 unigenes with an average length of 1056.41 bp and an N50 length of 2229 bp. The length distribution of transcripts and unigenes is shown in Table 1 and Figure S1. These results showed that the data were of high quality and the unigenes qualified for further annotation analysis. The raw data files have been uploaded to the NCBI sequence read archive (BioProject accession number: PRJNA508983).

Table 1 The length distribution of assembled transcripts and unigenes.

Length (bp)	Transcripts	Unigenes
Total Number	78138	59218
Largest Length	34133	34133
Smallest Length	201	201
Mean Length	1147.85	1059.41
N50 Length	2379	2229
200-500	38002 (49%)	30731 (52%)
501-1000	16142 (21%)	12097 (20%)
1001-2000	11054 (15%)	7661 (13%)
>2000	12940 (16%)	8729 (15%)

Functional annotation and classification of unigenes

To investigate information on gene functions more comprehensively, a total of 57812 unigenes were annotated using six databases including Nr, Pfam, Swiss-prot, COG, GO, and KEGG. The results of annotation of the unigenes are as follows: 18388 (31.81%) unigenes were annotated in Nr; 14039 (24.28%) in Pfam; 11986 (20.73%) in Swiss-prot; 14479 (25.04%) in COG; 14129 (24.44%) in GO, and 9176 (15.87%) in KEGG (Figure 2 and Table S3).

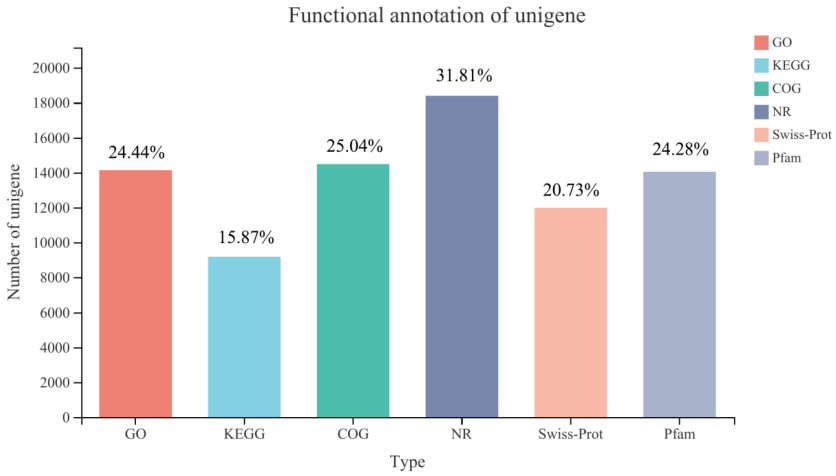

Figure 2 Number of annotated unigenes in 6 databases.

In the Nr database, the E -value distribution showed that 62.71% of unigenes exhibited homology ($<$ 1e-30) with previously reported sequences, and the similarity distribution showed that 82.65% of the unigenes were similar ($>$ 60%) to previous sequences (Figure 3A, B). The unigenes (12105, 65.20%) matched best matches to crustaceans, primarily including *Penaeus vannamei* (59.17%),*Hyalella azteca* (4.15%), and *Procambarus clarkii* (1.88%) (Figure 3C).

A total of 14,129 unigenes were classified into three categories, including biological processes (BP), cellular components (CC), and molecular function (MF) (Figure 4). Among the BPs, "cellular process", "metabolic process", and "biological regulation" were the top three functions. In CCs, "cell part", "membrane part", "organelle", "protein-containing complex", and "organelle part" were the dominant functions. Most unigenes were assigned to "binding" and "catalytic activity" among MFs. Moreover, we identified 83 unigenes related to olfactory and chemosensation by GO analysis (Table S4).

In the COG database, a total of 14,479 unigenes were assigned to 23 COG categories (Figure 5). As shown in Figure 5, category S formed the largest group which represented "function unknown" (8329 unigenes, 57.52%), followed by category O, representing "posttranslational modification, protein turnover, and chaperones" (1192 unigenes, 8.23%), and category J, representing "translation, ribosomal structure, and biogenesis" (1150 unigenes, 7.94%).

In our study, 9176 unigenes were assigned to 334 KEGG pathways and classified into six specific pathway groups (Figure 6). Among the six groups, the largest was "translation" (1,346 unigenes), followed by "signal transduction" (1,181), and "transport and catabolism" (761). These results can help elucidate the olfactory-related gene expression profile during the MP of crayfish and provide theoretical data for gene mining in crayfish.

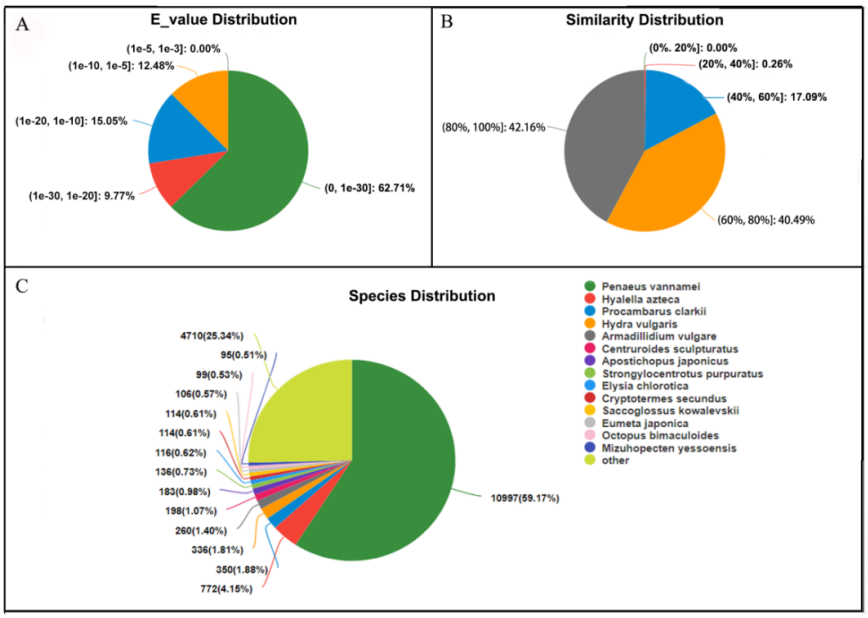

Figure 3 Pie charts for E-value (A), similarity (B), and species (C) distribution of unigenes based on the Nr database.

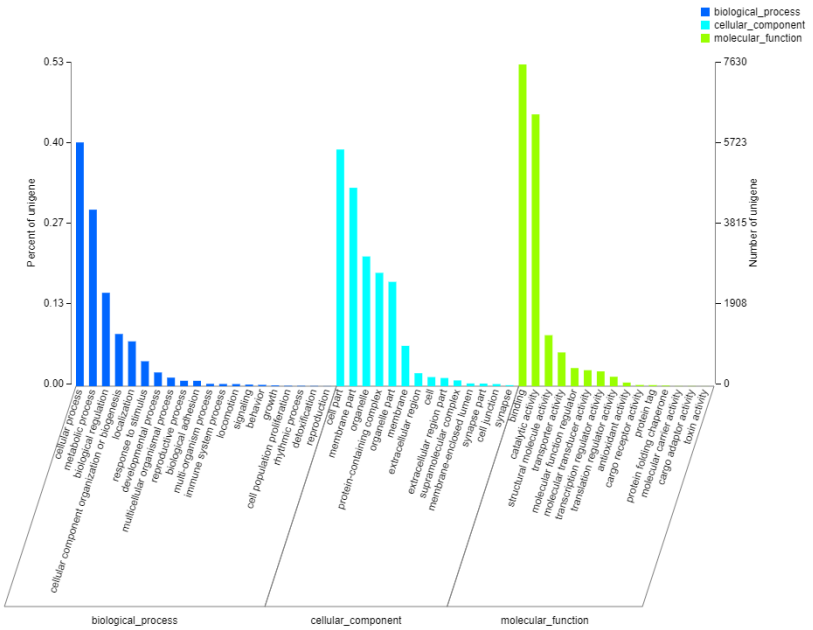

Figure 4 Gene Ontology (GO) classification of unigenes. All unigenes are annotated into 3 categories, namely biological process (BP), cellular component (CC), and molecular function (MF).

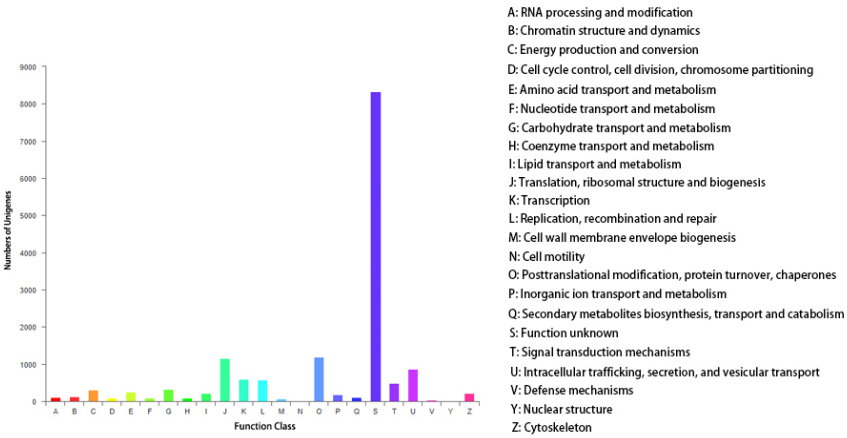

Figure 5 COG classification for the assembled unigenes. The X-axis indicates 23 COG categories and their detailed descriptions are presented on the right.

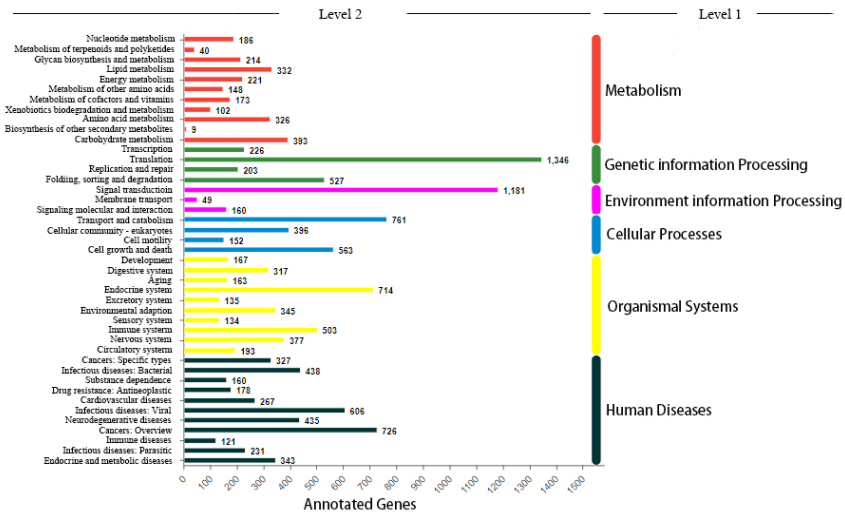

Figure 6 KEGG enrichment analysis for unigenes. The concrete description for the KEGG pathways (level 2) is listed on the left; the number at the end of each bar chart indicates the number of annotated unigenes. The descriptions of six major categories of KEEG terms (level 1) are listed on the right. The lines at the top of the figure show the KEGG level as well.

Identification and annotation analysis of DEGs

Based on the DESeq2 software, a total of 4889 DEGs with the FDR < 0.01 and $|$ log2 (Fold Change) $|$[?] 2 were identified between MP and NMP groups, including 2128 upregulated and 2761 downregulated unigenes (Figure S2). Furthermore, the annotations from the GO analysis of DEGs were classified into 44 GO categories, including the BP (18), CC (14), and MF (12). The top 3 GO terms in CC were cell part (GO:0044464), membrane part (GO:0044425), and protein-containing complex (GO:0032991); in BP were, cellular process (GO:0009987), metabolic process (GO:0008152), and biological regulation (GO:0065007); in MF were, binding (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198) (Figure 7).

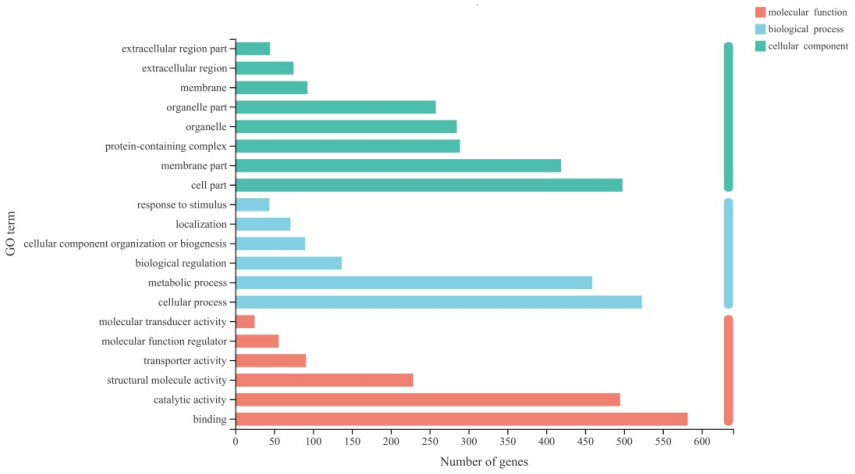

Figure 7 Classification of DEGs by GO annotation. The 3 categories were BP, CC, and MF.

Olfactory and chemosensory-related DEGs and olfactory transduction pathway

As shown in Table 2, 22 identified DEGs were related to olfactory and chemosensory functions. On comparing the MP and NMP groups, 9 DEGs were found to be downregulated, while 13 were upregulated, including 4 IRs or iGluRs, 8 G-protein coupled receptors, 5 transient receptor potential channels (TRP channels), 1 sodium-calcium exchanger, 1 olfactory receptor, 1 isomerase, and 2 chemosensory proteins (CSPs). In addition, the 9 sensory-related pathways and the number of DEGs are shown in Table 3.

Table 2 Olfactory and chemosensory regulation-related DEGs.

NCBI reference sequence	Gene ID	Description
AGJ51190.1	TRINITY_DN21918_c0_g1	olfactory ionotropic receptor IR93a
AAM47017.1	TRINITY_DN25415_c0_g2	ionotropic glutamate receptor subunit
ROT79299.1	TRINITY_DN20582_c0_g1	variant ionotropic glutamate receptor
XP_023236077.1	TRINITY_DN21176_c0_g4	peroxisomal acyl-coenzyme A oxidase 1-like
AGM39710.1	TRINITY_DN521_c1_g2	metal ion binding*
XP_027211194.1	TRINITY_DN1785_c0_g1	threonine-protein kinase 26-like*
AIT57587.1	TRINITY_DN3158_c0_g2	crustacean cardioactive peptide receptor, partial
XP_027208488.1	TRINITY_DN10908_c0_g3	G-protein coupled receptor GRL101-like
XP_021365014.1	TRINITY_DN28245_c0_g1	transient receptor potential cation channel subfamily A member 1-l
XP_027218241.1	TRINITY_DN518_c0_g1	chemosensory protein
XP_027236566.1	TRINITY_DN7269_c0_g2	chemosensory protein*
XP_027224784.1	TRINITY_DN16621_c0_g1	urotensin-2 receptor-like*
XP_018021398.1	TRINITY_DN22772_c0_g1	G-protein coupled receptor activity
PSN35490.1	TRINITY_DN22075_c0_g1	G-protein coupled receptor moody*
ROT70877.1	TRINITY_DN14467_c0_g4	Transient receptor potential channel pyrexia OS
ROT69786.1	TRINITY_DN24541_c0_g1	putative transient receptor potential cation channel subfamily A m
XP_027232413.1	TRINITY_DN4439_c0_g1	ankyrin-1-like*
XP_027231576.1	TRINITY_DN2621_c1_g1	transient receptor potential channel pyrexia-like
APG53786.1	TRINITY_DN5073_c0_g3	transient receptor potential cation channel pyrexia
XP_027234885.1	TRINITY_DN34955_c0_g1	olfactory receptor 4C6-like
AJO70196.1	TRINITY_DN32840_c0_g1	sodium-calcium exchanger 1*
ROT70691.1	TRINITY_DN4991_c0_g1	putative E3 ubiquitin-protein ligase TRIM32*

* represents the selected DEGs for RT-qPCR.

Table 3 Number of olfactory- and chemosensory-related DEGs enriched in different KEGG pathways.

Pathway ID	Description	Up number	Down number
map04740	Olfactory transduction	1	0
map04745	Phototransduction - fly	4	0
map04750	Inflammatory mediator regulation of TRP channels	4	1
map04024	cAMP signaling pathway	4	2
map04020	Calcium signaling pathway	6	3
map04010	MAPK signaling pathway	8	0
map04072	Phospholipase D signaling pathway	3	2
map04151	PI3K-Akt signaling pathway	7	4
map04014	Ras signaling pathway	5	2

Validation by RT-qPCR analysis

To verify the accuracy of the RNA-Seq data, 8 olfactory- and chemosensory-related genes were selected from among the DEGs and validated by RT-qPCR analysis. All selected DEGs were significantly expressed in the antennae. The results of RT-qPCR were consistent with those of RNA-Seq, indicating the reliability of the RNA-Seq data (Figure 8 and Table S1).

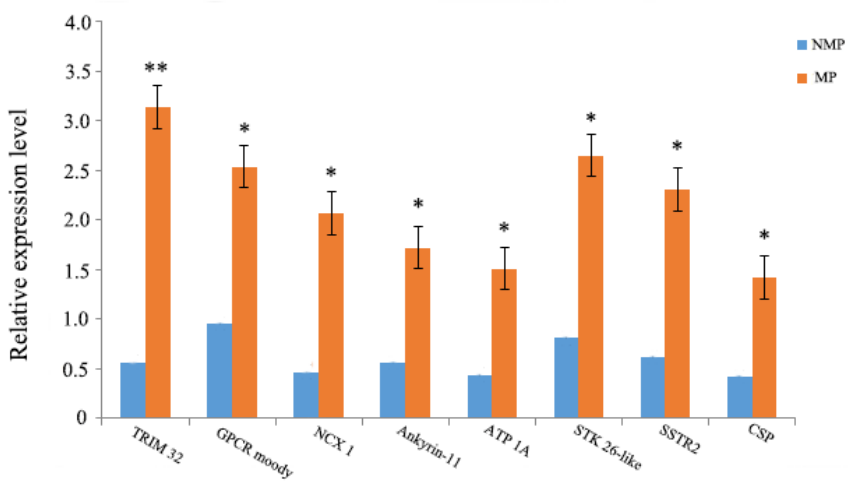

Figure 8 Relative levels of selected DEG expression by RT-qPCR and RNA-Seq. Levels are measured using the $2^{-\Delta\Delta^{c}T}$ method; β-actin is the internal normalization control. Data are shown as mean ± SD; * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

Numerous behavioral studies indicate that the male red swamp crayfish have "unidirectional receptors" for the sex pheromones released by females during the MP (Monteclaro et al., 2010; Oyama et al., 2020; Peddio et al., 2019). Male individuals can recognize different phases of females by detecting the pheromones and then performing courtship behaviors after checking the sex pheromone levels (Kubec et al., 2019). However, previous research suggests that the frequencies of copulation and oviposition in red swamp crayfish are significantly reduced when the temperature goes below 15 °C (Egly et al., 2019). Before RNA-Seq, we found that male red swamp crayfish were attracted to female conditioned water in MP but not in NMP.

We speculated that the expression of olfactory and chemosensory genes in antennae may be altered due to different temperatures and periods. In this study, we identified the olfactory or chemosensory genes and pathways in antennae of *P. clarkii* between MP vs. NMP by RNA-Seq for the first time. However, the information on olfactory and chemosensory genes was as sparse as expected, presumably since genetic annotation of species closely related to *P. clarkii* is scarce in public genetic databases; in general, the genetic information on olfaction in crustaceans is lacking. This study is a necessary supplement to the genetic information library for red swamp crayfish.

In this study, we identified a total of 22 DEGs related to "olfactory" or "chemosensory", including 4 "IRs or iGluRs", 8 "G-protein coupled receptor", 5 "transient receptor potential", 1 "sodium-calcium exchanger", and 2 "chemosensory proteins (CSPs)" but no insect olfactory proteins such as "odorant-binding proteins (OBPs)" or "odorant receptors (ORs)", consistent with results for other crustaceans (Groh et al., 2014; Kozma et al., 2020b; Kozma et al., 2018). Crustaceans and insects share a common ancestor; the latter evolved from ancestors and migrated from the sea to land, which required olfactory receptors for detecting signals in the air (Missbach et al., 2014). OBPs have been reported in insects and vertebrates (Pelosi et al., 2006; Sanchez-Gracia et al., 2009), and are suggested to adapt to the volatile signaling molecules and pheromones more effectively (Derby et al., 2016; Missbach et al., 2015). ORs are considered an adaptation to the terrestrial life of insects (Krang et al., 2012). Thus far, no study on crustaceans reports ORs, suggesting that these may be insect-specific (Missbach et al., 2014).

iGluRs mediate neuronal communication by forming glutamate ion channels in various animals (Benton et al., 2009). Generally, iGluRs assemble as heterotetramers, and each monomer of iGluRs has 4 major domains, namely amino-terminal domain (ATD), ligand-binding domain (LBD), common pore-forming transmembrane domain (TMD), and an intracellular C-terminal domain (CTD) (Reiner and Levitz, 2018). iGluRs can be divided into four well-known subfamilies as follows: kainate, delta, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs), and N-methyl-D-aspartate receptors (NMDARs), which perform various functions (Regan and Furukawa, 2016). Nevertheless, iGluRs are reportedly expressed in different parts of several crustaceans (Kozma et al., 2020a) but changes in their expressions are rarely reported. In the present study, several iGluRs were found to be downregulated after the crayfish entered the MP, which warrants further investigation into the phenomenon.

Ionotropic receptors (IRs) are a subset of ion channels that are gated by ligands (Harzsch and Krieger, 2018). These have been reported in crustaceans, chelicerates, nematodes, annelids, and gastropods (Derby et al., 2016). Corset et al., (2010) suggest that IRs may have evolved from non-NMDAR iGluRs in ancient protostomes (Croset et al., 2010). However, unlike iGluRs, only co-receptors, IR8a and IR25a, have 4 major domains while other IRs only have 3 domains (Derby et al., 2016). In this study, we found 6 types of IRs or IR-like, including IR93a, IR4, IR7, IR8a, IR21a-like, and IR25a-like. Three co-receptors (IR25a, IR8a, and IR93a) were identified in our study but not co-receptor IR76b, which is present in different organs of red swamp crayfish (Kozma et al., 2020a). Furthermore, we focused on changes in the gene expressions. Notably, of these related genes, all genes annotated to IR93a were downregulated when the crayfish entered the MP. IR93a is one of the co-receptors of the IR family, which is involved in the regulation of humidity and thermal perception in insects (Enjin et al., 2016; Knecht et al., 2017). IR93a in *Drosophilid* interacts with IR25a and exhibits distinct functions when bound to different IRs (Knecht et al., 2017). A colder aquatic environment may be a dominant factor that forces the crayfish to enter into NMP with less movement. During this period, survival is essential rather thanreproduction. Therefore, perception of humidity and temperature is more important as the crayfish will then transfer to subterranean burrows rather than the water bottom (Yoder et al., 2016). As a co-receptor, the interactions of IR93a and other IRs are rarely reported, and little information is available on controlling IR expression. No evidence suggests that IRs convey negative signals to inhibit the expression of other IRs and we speculated that the expression of IR may be controlled by the association of specific combinations of transcription factors with these sequences (Rytz et al., 2013).

Notably, DEGs related to the TRP channels were typically upregulated. TRP channels are homotetramers or heterotetramers with six transmembrane segments. There are eight subfamilies of TRP channels, including

TRPC, TRPA, TRPP, TRPN, TRPV, TRPM, TRPM, and TRPT (Van den Eynde et al., 2021; Venkatachalam et al., 2014; Venkatachalam and Montell, 2007). In the present study, six types of TRP channels (TRPA1, Pyrexia TRPA, TRPM2, TRPM3, TRPC4, and TRPV5) were identified. TRP channels participate in many sensory processes, such as vision, olfactory, audition, and temperature sensation, which affect the behaviors of creatures profoundly (Fowler and Montell, 2013). As the climate becomes warmer, more movements are generated by benthos and crayfish (Johnson et al., 2014; Larson and Magoulick, 2011; Wittwer et al., 2018). We speculated that the crayfish could enhance self-abilities for various senses instinctively for environmental adaptation.

A total of 3 and 5 GPCRs were up and downregulated, respectively. GPCRs are the most abundant cell surface receptors in the mammalian genome, accounting for more than 1 % of the human genome (Lander et al., 2001). In crustaceans, GPCRs are widely known to regulate physiological processes such as neuromodulation (Rump et al., 2021), reproduction (Tu et al., 2021), and neuropeptide conduction (Bao et al., 2018). Rump et al. (2021) inferred that GPCRs mediate olfactory sensation in four decapod crustaceans and suggested their function as putative chemosensory receptors (Rump et al., 2021). Based on our results, we reasonably suggest GPCRs may be a fundamental class of signaling receptors rather than pheromone-specific receptors. In addition, only one GR was identified in our study, and its expression was higher relative to other unigenes even though there was no significant difference between NMP and MP groups. GRs are reportedly widely expressed in legs and mouthparts of insects but are rarely found in crustaceans. Only the water flea, *Daphnia pulex,* a branchiopod crustacean, has 58 GRs, while other studies show that only a few GRs or GRLs are expressed in the antennae of crustaceans *Panulirus argus*(1 GR) (Kozma et al., 2020b), *Homarus americanus* (4 GRLs), and*Callinectes sapidus* (1 GR) (Kozma et al., 2020a). Our results further show the scarcity of GRs in decapod crustaceans, which indicated that these may not be the dominant chemosensory receptors in decapod antennae.

Surprisingly, two CSPs were up-regulated and annotated in the Pfam database to "insect pheromone-binding family, A10/OS-D". Their expressions were significantly higher in the MP group than in the NMP group. In addition, the expressions of OS-D proteins were also much higher relative to other unigenes. The OS-D gene family in insects are chemosensory proteins (CSPs), which possess a group of hydrophobic binding pockets (Wanner et al., 2004). The size, solubility, and overall structure of CSPs are similar to those of OBPs (Rothemund et al., 1999) but CSPs are less specific and more highly conserved (Jacquin-Joly et al., 2001). Previous studies support that CSPs are sensory-related proteins with pheromone binding function in insects (Bohbot et al., 1998; Jacquin-Joly et al., 2001). Unlike insect-specific proteins (OBPs), CSPs are found in crustaceans such as *Daphnia carinata* , and are expressed in ovaries, thoracic limbs, rectum, and second antennae in both sexual and asexual females (Li et al., 2016). This suggests that the CSPs may respond to environmental signals and control the reproductive switch from sexual to asexual reproduction in *D. carinata* (Li et al., 2016). Generally, owing to the lack of OBPs, crustaceans only have 0-2 CSPs. (Derby et al., 2016). Our results were consistent. Even though CSPs are broadly reported in insects, crustaceans, and myriapods (Chipman et al., 2014; Pelosi et al., 2014; Zhou et al., 2006), their functions in crustaceans remain unknown. Based on our results, we speculated that CSPs may be one of the dominant signaling receptors of pheromone binding in male red swamp crayfish. More attention to the expressions and changes of CSPs is needed in further studies.

CONCLUSION

In this study, RNA-Seq technology was used to analyze the antennae transcriptome of male red swamp crayfish between MP and NMP. A total of 13 upregulated and 9 downregulated DEGs were associated with olfactory- and chemosensory-related functions, of which 2 related to CSPs were remarkably upregulated after the red swamp crayfish entered the MP. Based on the levels of CSP-related DEG expression, we suggest that CSP may be the key receptor for chemical signals or sex pheromone reception in the antennae of red swamp crayfish. The results presented herein will be fundamental for future functional studies on olfactory and chemosensory genes in *P. clarkii* . The findings are expected to clarify the olfactory and chemical communication mechanisms in *P. clarkii* and provide new targets for invasion management in the future.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA ACCESSIBILITY

The raw data files have been uploaded to the National Center for Biotechnology Information (NCBI) sequence read archive (BioProject accession number: PRJNA508983).

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