RNA-Seq analysis of *Polyrhachis vicina* Roger and insights into the heat shock protein 90 and 70 families

JuanJuan Zhang¹ · GengSi Xi¹ · ZhiYi Guo¹ · FengHua Jia¹

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Abstract

The heat shock protein 90 (Hsp90) and heat shock cognate proteins (Hsc70) have been identified as chaperones of the ecdysone receptor (EcR)/ultraspiracle protein (USP) heterocomplex. However, little is known about the status of Hsp90 and Hsc70 in *Polyrhachis vicina* Roger. Here, we sequenced the transcriptomes of adult ants in *P. vicina* for the first time. Clean reads in female, male, and worker ants were annotated into 40,147 transcripts, and 37,488, 28,300, and 33,638 unigenes were assembled in female, male, and worker ants, respectively. According to RPKM, the numbers of differentially expressed genes between female and male ants, between female and worker ants, and between male and worker ants and the common differentially expressed genes were 12,657, 21,630, 15,112 and 3704, respectively. These results reveal that caste differentiation, caste specificity formation, and social divisions of *P. vicina* ants may be due to gene expression differences. Moreover, *PvEcR* and *PvUSP* were also detected as differentially expressed genes in the ants; specifically, *PvUSP* expression was higher than *PvEcR* expression in all castes. We speculate that *PvUSP* may have a role similar to that of juvenile hormone receptor. Four identified *PvHsp90* family members and 23 identified *PvHsp70* family members were found in the ants, and 2 *PvHsp90* genes and 8 *PvHsp70* genes were analyzed by qRT-PCR. Among those genes, the expression of 2 *PvHsp90* genes and 5 *PvHsp70* genes coincided with the expression profiles of *PvEcR* and *PvUSP*, which suggest that the characterization of *PvHsp90* and *PvHsc70* may be as EcR/USP molecular chaperones in *P. vicina*.

Keywords *Polyrhachis vicina* Roger · Transcriptome · Heat shock protein 90 family · Heat shock protein 70 family · Differentially expressed genes

Introduction

Ecdysone and juvenile hormone are important hormones that participate in insect growth and development. A chaperone is required to activate the DNA-binding activities of the ecdysone receptor (EcR)/ultraspiracle protein (USP) heterocomplex (Arbeitman and Hogness 2000). The activation of the EcR/USP heterocomplex initiates a cascade

ecdysone-responsive gene expression that leads to drastic changes in the differentiation of adult tissues and larval organs. Two proteins, heat shock protein 90 (Hsp90) and heat shock cognate proteins (Hsc70), have been found as chaperones of the heterocomplex.

Heat shock proteins (Hsps) are highly conserved proteins that are present in almost all living organisms. Hsps act as molecular chaperones, promoting correct refolding of denatured proteins and preventing protein aggregation in response to various environmental stress factors, such as extreme temperatures, starvation, ultraviolet radiation, heavy metals, and anoxia (Cheng et al. 2015). Hsps are involved in various physiological processes, including insect development, diapause, fecundity, and larva-to-adult survival, and in normal cellular activities such as protein trafficking, signal transduction, and protein-protein interactions. The genes that encode Hsps are also thought to be immunity-related genes (Tsan and Gao 2004) and play important roles in the safe and effective treatment of human diseases (Murshid et al. 2011).

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✉ GengSi Xi
xigengsi@snnu.edu.cn

¹ Institute of Zoology, College of Life Science, Shaanxi Normal University, Xi'an 710119, Shaanxi Province, People's Republic of China

Based on molecular weight and sequence homology, Hsps are divided into the Hsp90, Hsp70, Hsp60, and small Hsp families. There are four different types of Hsp90 proteins that exist in eukaryotes: chloroplast Hsp90, mitochondrial Hsp90, cytosolic Hsp90, and endoplasmic reticulum-based Hsp90 (ER-based Hsp90) (Johnson 2012). These diverse Hsp90 homologs appear to cooperate with other chaperone molecules, such as Hsp70 and Hsp40, to mediate the folding, stability, and transport of proteins as well as the assembly of proteins into multi-protein complexes (Johnson and Brown 2009; Marzec et al. 2012). The Hsp70 family represents one of the most highly conserved proteins identified in all organisms. The Hsp70 protein family includes heat shock inducible proteins (Hsp70) and heat shock cognate proteins (Hsc70) (Mayer and Bukau 2005). These two proteins have a high degree of sequence homology (Prentice et al. 2004; Shiota et al. 2010). The Hsp70 family is a supergene family, and it is well-known that Hsp70, Hsc70, and Hsp90 play important roles in the folding and maturation of steroid hormone receptors and different transcription factors (Wegele et al. 2004). Key members of the Hsp70 family have been identified in a few species of Hymenoptera (Elekonich 2009; Wang et al. 2012; Xu et al. 2010). However, we know little about the status of the Hsp90 and Hsc70 chaperone proteins in *Polyrhachis vicina* Roger ants.

P. vicina, as a typical eusocial insect, has the following characteristics: social division of labor, cooperative brood care, and generation overlap (Cardinal and Danforth 2011; Pockley 2003). *P. vicina* as a complete metamorphosis insect includes four stages: egg, larvae, pupa, and adult. Adult *P. vicina* can be divided into three castes: female, male, and worker. Recent studies showed that genetic factors influence the caste determination of social insects (Schwander et al. 2010), but a whole-genome sequence of *P. vicina* has not yet been obtained. In the present study, we used RNA-Seq to identify caste-specific genes by building transcriptomes of mated *P. vicina* females and males as well as *P. vicina* workers. We identified differentially expressed genes in female, male, and worker ants by comparative transcriptome analysis. We also screened *PvEcR* and *PvUSP*, the *heat shock protein 90* family and *heat shock protein 70* family homologs in *P. vicina* ants. Four *PvHsp90* unigenes and 18 *PvHsp70* unigenes were identified from the transcriptome of *P. vicina*, and their mRNA expression levels were examined using a tag-based digital gene expression (DGE) technique. Moreover, 2 significantly differentially expressed *PvHsp90* unigenes and 8 significantly differentially expressed *PvHsp70* unigenes in female, male, and worker ants were tested for transcriptome data validation by real-time quantitative PCR. Our data provide a comprehensive resource for exploring the molecular mechanism of social insect caste development, differentiation, and specificity formation and establish an essential genomic resource for further studies in this ant.

Methods

Ants

Colonies of *P. vicina* were purchased from Nanrui Ant Aquaculture center of Ruian, Zhejiang Province, China. Specimens of *P. vicina* were reared on 12 cm × 6 cm × 3 cm plastic containers under stable 16:8-h light:dark photoperiod at 26 ± 3 °C and 35–45% relative humidity in the Laboratory of Animal Reproduction and Development at the College of Life Science in the Shaanxi Normal University of Xi'an, China. *P. vicina* were fed a diet of a 300 mM sucrose solution. For different castes, 6 mated females, 20 males, and 20 workers separately were pooled together as a tissue source for current transcriptomic study. All the samples were frozen immediately in liquid nitrogen for 24 h and then delivered to the Gene Denovo Biotechnology Corporation (Guangzhou, China) on dry ice.

RNA extraction, library construction and sequence

Total RNAs were isolated separately using TRIzol reagent according to the manufacturer's protocol (TaKaRa, Dalian, China). RNA purity was checked using the NanoDrop 2000 spectrophotometer. And RNA integrity was assessed using the RNA Nano 6000 Assay kit of the Agilent Bioanalyzer 2100 system. After total RNA was extracted, eukaryotic mRNA was enriched with oligo(dT) beads, while prokaryotic mRNA was enriched by removing rRNA using a Ribo-Zero™ Magnetic Kit (Epicentre). Then, the enriched mRNA was fragmented into short fragments using fragmentation buffer and was reverse transcribed into cDNA using random primers. Second-strand cDNA was synthesized with a mixture containing DNA polymerase I, RNase H, dNTPs, and buffer. Then, the cDNA fragments were purified with a QiaQuick PCR extraction kit, end repair was performed, poly(A) was added, and the fragments were ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, PCR amplified, and sequenced carried out on an Illumina HiSeq™ 4000 platform using the 150-bp paired-end sequencing protocol of Illumina at Gene Denovo Biotechnology Co. (Guangzhou, China). Raw sequence data were deposited to the National Center for Biotechnology Information Short Read Archive (SRA) database under the accession number SRP151716.

De novo assembly

The raw reads were subjected to quality control to remove reads that contained adapters, reads in which more than 10% of the nucleotides (N) were unknown and read in which more than 40% of the bases were low quality (*Q* value ≤ 10). The Q20, Q30, and GC content of the clean reads were calculated.

Finally, the clean reads were selected for de novo assembly using a short reads assembling program—Trinity (Grabherr et al. 2011). The quality of transcript assembly was checked using parameters such as the N50 value, the transcript length, and the sequencing depth.

Annotation and classification of unigenes

Female, male, and worker ant transcriptomes were concatenated to create a combined transcriptome of *P. vicina*, which was used for BLASTx program searches with annotation against the NCBI non-redundant protein (Nr) database with an *E* value threshold of 1E−5. The unigene sequences were also aligned by BLASTx to sequences from protein databases such as the Swiss-Prot protein database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the COG/KOG database. Protein functional annotations were obtained according to the best alignment results. Advanced annotation of unigenes, including CDS prediction of the unigenes, SSR prediction, and Pfam protein domain prediction, was utilized to identify functional domains within the transcripts (Finn et al. 2014).

Quantification and differential expression analysis of unigenes

The expression of the unigenes was calculated and normalized to RPKM (reads per kb per million reads) (Mortazavi et al. 2008). The following formula was used: $RPKM = (1,000,000 \times C) / (N \times L)$, where *C* is the number of reads that are uniquely mapped to unigene A, *N* is the total number of reads that are uniquely mapped to all unigenes, and *L* is the length (base number) of unigene A.

To identify differentially expressed genes (DEGs) across samples or groups, the edgeR package (<http://www.r-project.org/>) was used (Robinson et al. 2010). *P* values were adjusted for multiple testing using the Benjamini-Hochberg method (Hochberg and Benjamini 1990). We identified genes with a fold change > 2 and a false discovery rate (FDR) < 0.05 in a comparison as significant DEGs. DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways.

qPCR

Twelve transcripts with significant differences among female, male, and worker ants were selected for experimental

Table 1 Primer information used in the real-time quantitative PCR

Unigene (gene)	Primer sequence (5'→3')	Tm (°C)	Product size (bp)
Unigene0000981 (Hsp70)	GTGTTTTGGGGAATCTCACTCC TATTGTTTCGCCTGGTGTTCCTA	60	144
Unigene0001394 (Hsc70-5)	GATAAAACACCACCTTGAACAGCA TTTAGTGGGTGGACAAACTCGTAT	60	135
Unigene0005056 (Hsc70-5)	AAATCTGAAGGTGTAAAAGGTGCAG AACATAAGACGGTGTGGTTCTAGAA	60	135
Unigene0010921 (Hsp70)	ACAAACACGTAATCAAGCAGATCA TCACTCTTCACAACAGATTCCAAA	60	92
Unigene0012706 (Hsc70-3)	TTTGAAGCTAAATGTGCAGTGGG AATCTGACAAAACATCCGGCATC	60	114
Unigene0014930 (Hsc70-4)	CAAGAGGAATACAACGATACCAACG TATTGTTATCCTTGGTCATCGCTCT	60	122
Unigene0017805 (Hsp70-4L)	CCGTATCCTTTGAAACTAACATGGG CGAACGATAGAACGTCAACATCTTT	60	114
Unigene0023392 (Hsp70)	ATCGGCTATGACTCCGTCTTTC GTGGTTGCTATTTCGTCAAGATCG	60	101
Unigene0012302 (Hsp83)	GCAAAGTCATGAAAGAGATCCTGG TCCCATAGTGGATGTATCTCTGAGA	60	163
Unigene0019217 (Hsp90)	GGATTCTGGTATTGGCATGACAAA TATCATTCATATCCTGAGCGTTCTGA	60	125
Unigene0020654 (EcR)	GGTACGATCTTCGCTAAACTCCT GAAAAGTGGCAGCTTCTTGTCT	60	114
Unigene0018981 (USP)	CTGGCTGGAACGAATTACTA GCATCTTCGACACTAGTTCT	60	168
RPL-18	TTCAAAAATCCTTGCATCGTCAGT CACCTATCTCACTTGCACGTATTG	60	107
β-Actin	CCCTCTTCCAACCTTCCTTC CCGCCGATCCATACGGAATA	60	250

validation of the transcriptome assembly using qPCR. Two of the transcripts belonged to the Hsp90 family, and eight transcripts belonged to the Hsp70 family. Total RNA from each sample was extracted from 2 mixed mated females, 8 mixed males, and 6 mixed workers, respectively. Transcript-specific primers were designed by Primer3 plus and verified by NCBI blast to cross-check their specificity prior to experimentation. The primer sequences used are listed in Table 1. qPCR reactions were performed with a 25 µl total reaction volume including 12.5 µl of 2× SYBR Premix Ex Taq™ master mix (TaKaRa, China), 10.5 µl of ddH₂O, 0.5 µl of each of gene-specific primer (10 µM), and 1 µl of cDNA template. The qPCR reactions were performed using a CFX96 real-time system (Bio-Rad) using the following parameters: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s, and finally 56 °C for 60 s. A melting curve analysis from 52 to 95 °C was performed to ensure the consistency and specificity of the amplified product. The mRNA quantity of each unigene was calculated with the $2^{-\Delta\Delta C_t}$ method (Applied Biosystems, user bulletin no. 2) (Livak and Schmittgen 2001), and gene expression levels were normalized against the corresponding β -actin and rpl-18 expression levels. For all the qPCR experiments, three biological replicates were conducted.

Results

Illumina sequencing and de novo assembly of the *P. vicina* transcriptome

The transcriptome of female, male, and worker *P. vicina* ants was assembled de novo using paired-end raw reads generated by Illumina Hiseq™ 2000. An overview of the sequencing and assembly is outlined in Table 2. After filtering the low-quality and adapter raw reads, the numbers of high-quality clean reads in the female, male, and worker *P. vicina* ants were 58,021,092, 40,121,968, and 46,215,280, respectively. All clean reads were assembled into transcripts with short reads using the assembling program Trinity (Grabherr et al. 2011). In total, 40,147 unigenes were assembled, the maximum length of a transcript was 28,568, the minimum length was 201, and the average length was 955; the N50 value of

the transcriptomes was 2157, and the transcript length distribution is shown in Fig. S1.

Annotation and classification of unigenes

The *P. vicina* unigenes were prepared for annotation using the Nr, Swiss-Prot, KOG, KEGG, GO, and Pfam databases so that annotation of the maximum number of transcripts could be possible. The numbers of unigenes annotated as female, male, and worker were 37,488, 28,300, and 33,638, respectively (Table 2). Of the 40,147 unigenes, we were able to annotate 20,375 in four databases, while 19,772 of the unigenes remained unannotated (Table S1). Among them, the number of unigenes annotated in Nr, Swiss-Prot, KOG, and KEGG databases was 19,342, 14,068, 10,861, and 8620, respectively (Table S1).

Using the BLASTx program to compare the assembled unigene sequence with the Nr database (19,342), the *E* value distribution of the top hits in the Nr database showed that 57.59% of the mapped sequences had strong homology ($< 1E-50$), whereas 42.41% of the homology sequences ranged between $1E-05$ to $1E-50$ (Fig. S2a). For species distribution, organismal annotation data count of annotated unigenes showed that *Camponotus floridanus* (53%, 10,230 unigenes) was the top organisms sharing homology with *P. vicina* (Fig. S2b).

Differentially expressed genes

The functional annotation and classification of the collective transcriptome provided us an understanding of the overall transcriptomic landscape of *P. vicina*. The abundance of all the genes (40147) was normalized and calculated by the reads per kilobase per million reads (RPKM) method using uniquely mapped reads (Mortazavi et al. 2008). Differentially expressed genes were set as the threshold, with conditions of a $\log_2$ fold change > 1 and a *P* value < 0.05 (Storey and Tibshirani 2003). The numbers of differentially expressed genes between female and male ants, between female and worker ants, and between male and worker ants were 12,657 (2594 up and 10,063 down), 21,630 (6224 up and 15,406 down), and 15,112 (6356 up and 8756 down), respectively (Fig. 1). There were 3704 common differentially expressed

Table 2 Summary of the sequence assembly after Illumina sequencing

Sample	Raw reads	Clean reads	Adapter (%)	Low quality (%)	Q20 (%)	Q30 (%)	GC content (%)	Genes Num
Female	60,016,466	58,021,092	45,574 (0.08%)	1,945,792 (3.24%)	96.41	90.83	43.90	37,488
Male	41,375,584	40,121,968	28,340 (0.07%)	1,221,852 (2.95%)	96.46	90.88	43.06	28,300
Worker	48,013,376	46,215,280	44,272 (0.09%)	1,750,612 (3.65%)	96.25	90.45	45.58	33,638

Fig. 1 Numbers of differentially expressed genes (DEGs). The number of DEGs are marked on each column. Numbers of upregulated unigenes are represented in red column, and downregulated ones in green column

genes among female, male, and worker ants (Fig. 2). In total, we found 5922 differentially expressed genes between female and male ants that were also differentially expressed between female and worker ants. Additionally, 7688 genes that were differentially expressed between female and worker ants were also differentially expressed between male and worker ants (Fig. 2). As shown in Fig. 2, 1885 differentially expressed genes between male and worker ants were also differentially expressed between female and male ants. These data show that the number of differentially expressed genes of female and worker ants is at the most, which may be implied that the degree of dimorphism between queens and workers is primarily influenced by genetic effects.

Fig. 2 Venn diagram of the number of differentially expressed genes in female, male, and worker ants. Red color means the number of differentially expressed genes in female and worker ants. Green color means the number of differentially expressed genes in female and male ants. Blue color means the number of differentially expressed genes in male and worker ants

Enriched GO and KEGG classes among differentially expressed unigenes

In total, 10,397 unigenes were annotated into 48 subcategories belonging to three main GO categories (Fig. S3). To analyze the gene functions of the differentially expressed unigenes, a GO enrichment analysis was performed using Fisher's exact test in Blast2GO. GO terms with a corrected P value < 0.05 were considered significantly enriched among the differentially expressed genes. The GO functional enrichment analysis of the 12,657 DEGs between female and male ants revealed significantly enriched terms in the biological processes and molecular functions categories (Fig. 3). Metabolic process (GO:0008152) with 1797 genes was dominant in the biological process category and binding (GO:0005488) with 2277 genes was dominant in molecular function category. In the analysis of the 21,630 DEGs between female and worker ants, metabolic process (GO:0008152) with 2860 genes was dominant in the biological process category and binding (GO:0005488) with 3461 genes had advantage in molecular function category. In the analysis of the 15,112 DEGs between male and worker ants, metabolic process (GO:0008152) with 2321 genes and catalytic binding (GO:0003824) with 2441 genes were the significantly enriched term. These results suggested that there may be more differences in protein synthesis events and metabolic activity among different caste ants.

Additionally, KEGG pathways enrichment analysis showed that 4313 unigenes could be mapped to the pathways, and we found that the basal transcription factors pathway (ko03022) was the only significantly enriched ($FDR < 0.05$) pathway for the DEGs between the male and worker ants (Fig. 4). And there were 12 pathways significantly enriched in DEGs between the female and worker, which was included ribosome biogenesis in eukaryotes (ko03008), mRNA

Fig. 3 Histogram of GO classifications of differentially expressed genes in female, male, and worker ants. **a** Histogram of GO classifications of differentially expressed genes between female and male ants. **b** Histogram of GO classifications of differentially expressed genes between female and worker ants. **c** Histogram of GO classifications of

differentially expressed genes between male and worker ants. Red color means significantly up-expressed genes in different GO classifications. Green color means significantly down-expressed genes in different GO classifications

Fig. 4 KEGG classifications of differentially expressed genes in female, male, and worker ants. **a** KEGG classifications of differentially expressed genes in female and male ants. **b** KEGG classifications of differentially expressed genes in male and worker ants. **c** KEGG classifications of differentially expressed genes in male and worker ants. Rich factor means the ratio of the number of genes that are differentially expressed

surveillance pathway (ko03015), nucleotide excision repair (ko03420), glutathione metabolism (ko00480), basal transcription factors (ko03022), endocytosis (ko04144), ubiquitin-mediated proteolysis (ko04120), RNA transport (ko03013), fanconi anemia pathway (ko03460), DNA replication (ko03030), dorso-ventral axis formation (ko04320), and base excision repair (ko03410). Seven pathways (glutathione metabolism, ribosome biogenesis in eukaryotes, nucleotide excision repair, DNA replication, dorso-ventral axis formation, RNA transport, mismatch repair) were enriched with the Q value, which was less than 0.05 in male as compared with the female ants. It suggested that in addition to the differences between the female and male castes, great changes have also occurred among the female and worker ants.

Expression profiles of *PvEcR*, *PvUSP*, and *PvHsp90* in different castes of adult *P. vicina*

To further understand the mechanism of caste differentiation, specifically formation based on interactions with the hormonal system, the differential expression of *PvEcR*, *PvUSP*, and *PvHsp90* was investigated (Fig. 5, Table S2), since their interaction of these genes is essential for the transcriptional response to ecdysteroids in insects. We first identified *PvEcR*, *PvUSP*, and *PvHsp90* unigenes through the Nr database, and four paralogs within the *PvHsp90* gene family were found (Fig. 6). Among them, *PvEcR*, *PvUSP*, and two members of the *PvHsp90* family were significantly differentially expressed in the different castes of adults and were highly expressed in female ants, moderately expressed in male ants, and moderately expressed at low levels in worker ants (Table 3 and Fig. 5). Additionally, we also found two DEG

genes located in the pathway gene to the total number of genes in the pathway, and the larger the rich factor is, the higher the degree of pathway enrichment. Q value is the P value after the correction of multiple hypothesis testing, which ranges from 0 to 1, and the closer to 0, the more significant the enrichment is

members of the Hsp90 family were distributed in the protein processing of endoplasmic reticulum (ko04141) (Table 5).

Fig. 5 Expression patterns of 29 selected genes in different caste ants. The heat map visualizes the expression of 4 members of *PvHsp90* family, 23 members of *PvHsp70* family, *PvEcR*, and *PvUSP*, using the RNA-Seq data derived from the three caste adult ants based on $\log_2$ fold change and FDR values (Tables 3, 4). The green bands indicate low gene expression quantity; the red bands indicate high gene expression quantity. Unigenes with significantly differentially expressed were highlighted in italic bold. Gene names were given to the unigenes by Blast searching for the closet homolog in GenBank. The unigenes indicated with an asterisk were further analyzed for their expression using real-time quantitative PCR

Fig. 6 Maximum likelihood phylogeny of heat shock protein 90 and 70 families in *P. vicina*. Phylogenetic tree of Hsp90 and 70 families was reconstructed with MEGA 7.0 software. The analysis of protein domains was performed by MEME 4.9.0 software

Expression profiles of the *PvHsp70* family

Twenty-three Hsp70 unigenes were identified from the transcriptome annotation by evolutionary relationship and domain analysis of Hsp70 gene family (Fig. 6). Phylogenetic reconstruction of Hsp70 indicates the higher the conservation of the eight key sequence motifs within these genes, the greater the expression level (Figs. 5, 6). Differentially expressed genes were then used to study the changes in the Hsp70 family gene expression of different caste adult ants in *P. vicina* (Tables 4 and S3 and Fig. 5). The mRNA levels of 15 Hsp70 genes changed significantly in different caste adults. The others, except for 3 Hsp70 (unigene0014930, unigene0027174, and unigene0037817), stayed at higher mRNA levels in male ants

(Fig. 5). Hsp70 (unigene0001394, unigene0026469, unigene0032497, unigene0026003, and unigene0038805) were the higher mRNA levels in worker ants genes (Fig. 5), whereas the other genes of Hsp70 (unigene0000981, unigene0005056, unigene0010921, unigene0012706, unigene0017805, unigene0023392, and unigene0035884) were the higher mRNA levels in female ants (Fig. 5). Additionally, through KEGG pathway enrichment analysis, we found 10 *PvHsp70* family members that were significantly differentially expressed genes in the different caste ants were assigned to 9 KEGG pathways (Table 5). Four significant DEGs (unigene0014930, unigene0026469, unigene0027174, and unigene0037817) were mainly enriched in four pathways (protein processing in endoplasmic reticulum (ko04141),

Table 3 Functional annotation and differential expression of *PvEcR*, *PvUSP*, and *PvHsp90* in different castes of *P. vicina*

Gene	FDR			Blast nr
	Female vs male	Female vs worker	Male vs worker	
Unigene0020654	<i>3.92E-40</i>	<i>2.48E-186</i>	<i>1.58E-43</i>	EcR-A mRNA for ecdysone receptor A isoform (AB296080.1)
Unigene0018981	<i>1.53E-23</i>	<i>4.57E-146</i>	<i>4.83E-40</i>	Retinoic acid receptor RXR (XM_011266830.2)
Unigene0012302	0	0	0	Heat shock protein 83 (XM_011252510.3)
Unigene0019217	0	0	<i>5.67E-321</i>	Heat shock protein 90 (hsp90) (KF922652.1)
Unigene0025963	0.81235781	1	1	Heat shock protein 90 alpha family class A member 1 (XM_016925810.2)
Unigene0032860	0.62550242	1	0.34829499	Heat shock protein 83 (XM_014429459.2)

FDR, false discovery rate. Significant differential expressions of genes between any two ants are shown in italics

Table 4 Functional annotation and differential expression of the *PvHsp70* family members in different caste of *P. vicina*

Gene	FDR			Blast nr
	Female vs male	Female vs worker	Male vs worker	
Unigene0000186	0.372501882	1	0.34829498	Heat shock protein (XM_007833041)
Unigene0000981	<i>3.67E-303</i>	<i>0</i>	<i>0.00049363</i>	Hypoxia up-regulated protein 1 (XM_011254377.3)
Unigene0001394	0.003157682	<i>5.26E-21</i>	<i>2.19E-27</i>	Heat shock protein cognate 5 (Hsc70-5) gene (JQ001506)
Unigene0005056	8.12E-46	<i>0</i>	<i>1.18E-214</i>	Heat shock 70-kDa protein cognate 5 (XM_025405926.1)
Unigene0010921	<i>0.002997530</i>	<i>0.044269312</i>	0.19098621	Molecular chaperone DnaK, partial
Unigene0012706	<i>0</i>	<i>0</i>	<i>7.57E-29</i>	Heat shock 70-kDa protein cognate 3 (XM_012376222.1)
Unigene0014930	<i>2.85E-256</i>	<i>6.24E-234</i>	<i>0</i>	Heat shock 70-kDa protein cognate 4 (MF596491.1)
Unigene0017805	<i>1.37E-208</i>	<i>0</i>	<i>1.57E-87</i>	Heat shock 70-kDa protein 4 L (XM_011257942.3)
Unigene0022901	1.16E-10	1.82E-136	1.33E-72	Heat shock 70-kDa protein cognate 4 (XM_011261684.3)
Unigene0023392	<i>2.15E-77</i>	<i>1.57E-34</i>	<i>3.47E-15</i>	Rod shape-determining protein MreB-like (XM_017230291.1)
Unigene0026003	No test	<i>4.36E-05</i>	<i>0.00082408</i>	Rod shape-determining protein MreB-like (XM_017230291.1)
Unigene0026469	No test	<i>0.014633838</i>	0.06067374	Molecular chaperone DnaK (dnaK) gene (FN666595.1)
Unigene0027042	0.115739934	1	0.19098621	Heat shock protein 70 (dnaK) gene (AY063744.1)
Unigene0027174	<i>0.036378821</i>	No test	<i>0.03329804</i>	Chaperone protein DnaK (dnaK) gene (KT874323.1)
Unigene0031020	0.209170745	1	0.19098621	Heat shock protein family A member 8 (XM_011542798.1)
Unigene0032497	No test	<i>8.42E-05</i>	<i>0.00152488</i>	Heat shock 70-kDa protein cognate, partial (EU514494.1)
Unigene0035884	<i>0.002997530</i>	<i>0.001173688</i>	No test	3-Oxoacyl-acyl-carrier-protein synthase, mitochondrial-like (XM_012006786.1)
Unigene0036501	0.625502418	0.170540515	1	Heat shock 70-kDa protein 1A (KY500386.1)
Unigene0036576	0.372501882	0.798002359	0.19098621	hsp70-like protein (XM_018894370.1)
Unigene0037817	<i>0.036378821</i>	No test	<i>0.03329804</i>	Heat shock protein 70 2 (XM_001482809.1)
Unigene0037819	1	0.094116953	0.34829499	—
Unigene0038805	0.625502418	<i>5.94E-05</i>	<i>0.00011651</i>	Rod shape-determining protein MreB-like (XM_017230291.1)

FDR, false discovery rate. Significant differential expressions of genes between any two ants are shown in italics

endocytosis (ko04144), spliceosome (ko03040), and longevity regulating pathway—multiple species (ko04213)). Besides that, Unigene0000981 and unigene0017805 also related to protein processing in endoplasmic reticulum (ko04141). Moreover, unigene0005056 and unigene0032497 were distributed in the RNA degradation pathway (ko03018). Unigene0035884 was found to be participated in fatty acid biosynthesis (ko00061), fatty acid metabolism (ko01212), and biotin metabolism (ko00780), and unigene0012706 was found to participate in protein processing of endoplasmic reticulum (ko04141) and protein export (ko03060) pathways (Table 5). Those indicated that Hsp70 family likely contributed to development and metabolism, stress response, and longevity regulated in *P. vicina*.

Experimental validation of RNA-Seq data by quantitative real-time PCR

For validation of the differential gene expression results by RNA-Seq analysis, we selected 12 significantly differentially expressed genes shown in Tables 3 and 4 and then performed quantitative real-time PCR (qRT-PCR). The qRT-PCR results

are shown in Fig. 7. The qRT-PCR expression results were almost the same as the results obtained from the Illumina sequencing data and verified the validity of the expression data. The qRT-PCR results revealed that *PvEcR*, *PvUSP*, 2 *PvHsp90s*, and 6 *PvHsp70s* transcripts that were demonstrated to have higher expressions in female ants by RNA-Seq were confirmed by qPCR (Fig. 7). Additionally, RNA-Seq data for Unigene0014930 was richer in male ants, which is consistent with the qPCR results (Fig. 7). For Unigene0001394, the qRT-PCR results were not consistent with the results of the transcriptome in different ant castes; the qRT-PCR results showed that Unigene0001394 was richer in female ants (Fig. 7).

Discussion

In the present study, the transcriptomes of adult *P. vicina*, including mated female, male, and worker ants, were established for the first time, which revealed the genetic structure of *P. vicina* and provided a relatively comprehensive gene

Table 5 Enriched pathways of the *PvHsp90* and *PvHsp70* family members in different caste of *P. vicina*

KEGG_A_class	Pathway	Pathway ID	Genes
Genetic information processing	Protein processing in endoplasmic reticulum	ko04141	<i>Unigene0000981</i> , <i>Unigene0012302</i> , <i>Unigene0012706</i> , <i>Unigene0014930</i> , <i>Unigene0017805</i> , <i>Unigene0019217</i> , <i>Unigene0022901</i> , <i>Unigene0025963</i> , <i>Unigene0026469</i> , <i>Unigene0027174</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i> , <i>Unigene0037817</i>
Metabolism	Fatty acid metabolism	ko01212	<i>Unigene0035884</i>
Cellular processes	Endocytosis	ko04144	<i>Unigene0014930</i> , <i>Unigene0022901</i> , <i>Unigene0026469</i> , <i>Unigene0027174</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i> , <i>Unigene0037817</i>
Genetic information processing	Spliceosome	ko03040	<i>Unigene0014930</i> , <i>Unigene0022901</i> , <i>Unigene0026469</i> , <i>Unigene0027174</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i> , <i>Unigene0037817</i>
Metabolism	Fatty acid biosynthesis	ko00061	<i>Unigene0035884</i>
Genetic information processing	RNA degradation	ko03018	<i>Unigene0000186</i> , <i>Unigene0005056</i> , <i>Unigene0027042</i> , <i>Unigene0032497</i>
Organismal systems	Longevity regulating pathway—multiple species	ko04213	<i>Unigene0014930</i> , <i>Unigene0022901</i> , <i>Unigene0026469</i> , <i>Unigene0027174</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i> , <i>Unigene0037817</i>
Genetic information processing	Protein export	ko03060	<i>Unigene0012706</i>
Metabolism	Biotin metabolism	ko00780	<i>Unigene0035884</i>
Organismal systems	Antigen processing and presentation	ko04612	<i>Unigene0025963</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i>
Environmental information processing	PI3K-Akt signaling pathway	ko04151	<i>Unigene0025963</i>
Organismal systems	Estrogen signaling pathway	ko04915	<i>Unigene0025963</i> , <i>Unigene0031020</i> , <i>Unigene0036501</i>
Environmental information processing	MAPK signaling pathway	ko04010	<i>Unigene0031020</i> , <i>Unigene0036501</i>
Organismal systems	Progesterone-mediated oocyte maturation	ko04914	<i>Unigene0025963</i>
Organismal systems	NOD-like receptor signaling pathway	ko04621	<i>Unigene0025963</i>

Significant differential expressions of genes in KEGG pathway are shown in italics

Fig. 7 Quantitative real-time PCR validation of the differentially expressed genes in female, male, and worker ants. The data are the average $\pm$ standard error of three independent replicated qPCR

experiments. Significant differential expressions of genes between any two ants were identified with one-way analysis of variance analysis and were marked by an asterisk, * $p < 0.05$, ** $p < 0.01$

pool of *P. vicina* to deeply explore development, caste differentiation, and specificity formation of this ant species.

In total, 12,657, 21,630, and 15,112 differentially expressed genes were detected between female and male ants, between female and worker ants, and between male and worker ants, respectively (Fig. 7). The numbers of differentially expressed genes showed a variety of genetic differences among the different castes of adult ants. Although female and worker ants are of the same sex, the largest expression difference existed between them and based on the GO and KEGG enrichment analyses showed that the DEGs involved in DNA replication process, development, signal transduction, and immune defensive, which may be related with of the social division of labor in nests. The lowest expression difference was observed between female and male ants, and through KEGG analysis based on gene functional annotation, the DEGs mainly associated with metabolism and development. Although they are of different sexes, they all bear the same responsibility regarding reproduction. We suggest that caste differentiation, specificity formation, and social division are related to the physiological metabolic activities of different castes, which can be ascribed to differential gene regulation.

The expression profiles of *PvEcR* and *PvUSP* were different in the different castes of *P. vicina*, but the expression difference of those two genes was consistent in the different caste adults, i.e., the highest gene expression profile according to quantity was observed in female ants, followed by that in male and worker ants (Fig. 5 and Table S2). The highest expression of *PvEcR* was observed in female ants, and this expression was twice as high as that in male ants and 12 times higher than that in the worker ants. The expression of *PvUSP* in female ants was 1.5 times higher than that in male ants and 3 times higher than that in worker ants. Usually, EcR and USP form a heterodimeric complex and are bound by 20-hydroxyecdysone (20E) to activate the expression of 20E-response genes and regulate the growth, development, and reproduction of insects (Cao et al. 2015; Yan et al. 2016; Zou et al. 2017). Here, we found that *PvEcR* and *PvUSP* may be regulated caste specificity formation in *P. vicina*

adults. The higher expression of *PvEcR* and *PvUSP* in the female ants may be related to vitellogenesis (Planello et al. 2015). On the other hand, EcR and USP expressions may reflect 20E levels (Li et al. 2014). According to this proposal, our investigations may be indirectly reflected that the level of 20E in male ants is lower than that in female ants, which agrees with the conclusions of Handler (1982), Hodgetts et al. (1977), and Schwedes and Carney (2012). In our results, *PvUSP* gene expression was higher than *PvEcR* expression in all castes, which indicated that *PvUSP* not only participates in the 20E pathway but also may play roles in regulating other pathways that are important for physiological activities in ants. Guo et al. (2012) reported that Met is involved in 20E function and interacts with EcR-USP in physiological activities (Guo et al. 2012). Met was identified as a JH receptor (Gujar and Palli 2016). JH belongs to a very important group of steroid hormones in insects that regulates the diverse physiological processes of insects throughout the developmental stages (Hepat and Kim 2014). We did not find the *PvMet* gene in the sequenced transcriptomes of adult *P. vicina*. Another JH receptor is USP (Jones and Sharp 1997; Jones et al. 2001). Therefore, we hypothesized that *PvUSP* may function as a JH receptor in *P. vicina*, which needs to require further verification by experiment.

A functional relationship between steroid hormones and HSPs has been reported, and it is known that Hsp70, Hsc70, and Hsp90 play important roles in the folding and maturation of steroid hormone receptors and different transcription factors (Wegele et al. 2004). Studies of *Drosophila* EcR/USP DNA-binding activity have demonstrated that Hsp90 and Hsc70 act as molecular chaperones associated with EcR/USP (Arbeitman and Hogness 2000). There are four Hsp90 family members in *P. vicina* adults (Fig. 5 and Table 3), and based on comparative homologies with other species, Unigene0012302 showed 99% identities to Hsp83 of *Camponotus floridanus* (XP_011250812.1), and unigene0019217 was 67% identities to heat shock protein 90 of *Scylla paramamosain* (AGC54636.1). The expression levels of Unigene0012302 and Unigene0019217 are highly

consistent with *PvEcR* and *PvUSP* expression levels in caste specificity, i.e., the highest expression level was observed in female ants, followed by male and worker ants, which was verified by qRT-PCR analysis (Fig. 7). This result may further provide evidence that Hsp90 family members may be as molecular chaperones of *PvEcR* and *PvUSP*, which needs to be further verified by experiments. Will et al. (2017) report Hsp83 as a chaperone homologous to Hsp90 that impacts the lifespan, fecundity, and embryogenesis of *Drosophila melanogaster* and *Acyrthosiphon pisum* (Will et al. 2017). In our study, Unigene0012302 was expressed in greater quantities than Unigene0019217 in the female and male ants. We speculate Unigene0012302 plays significant roles in regulating the growth and development of *P. vicina*.

The family includes cognate proteins (Hsc70) and inducible members (Hsp70). The transcriptomes of Hsp70 family members in the different castes of *P. vicina* were detected. In *P. vicina*, 23 Hsp70 family members were identified through the Nr database, 15 members (unigene0000981, unigene0001394, unigene005056, unigene0010921, unigene0012706, unigene0014930, unigene0017805, unigene0023392, unigene0026003, unigene0026469, unigene0027174, unigene0032497, unigene0035884, unigene0037817, and unigene0038805) were significantly differentially expressed among the castes, and 7 members (unigene0026003, unigene0026469, unigene0027174, unigene0032497, unigene0035884, unigene0037817, and unigene0038805) showed very low expression levels (Fig. 5 and Table S3). Thus, 8 members of Hsp70 family were selected for qRT-PCR analysis. The results showed that the expression of 5 members coincided with the *PvEcR*/*PvUSP* expression profiles, which implied that those Hsp70 family members may be molecular chaperones of *PvEcR*/*PvUSP* (Fig. 7). Eight Hsp70 family members exhibited higher expression levels in female ants than in worker ants, which may suggest the female ants' status and abilities to respond to variable environments in the ant colony. This may relate to Hsp70 family members' diversity modulation mechanism.

The highest RPKM value was observed for Unigene0012706 (Fig. 5), which showed 97% identity to *Monomorium pharaonis* heat shock 70-kDa protein cognate 3 (XP_012525877.1) and 96% identity to *Wasmannia auropunctata* heat shock 70-kDa protein cognate 3 (XP_011708166.1). *Drosophila* Hsc70-3, as an ER-resident molecular chaperone, recognizes misfolded proteins in the ER lumen (Rubin et al. 1993). Based on this information, we hypothesized that Unigene0012706, as molecular chaperone, would have this same function.

Only Unigene0014930 (Fig. 5), a *PvHsp70* family member, was expressed at higher levels in the male ants than in female and worker ants. According to the results of the NCBI comparison, Unigene0014930 showed 98% identity to *Camponotus floridanus* heat shock 70-kDa protein cognate

4 (XP_011268514.1), 97% identity to *Atta cephalotes* and *Pogonomyrmex barbatus* heat shock 70-kDa protein cognate 4 (XP_012063429.1). Hsc70-4 of *Drosophila melanogaster* is expressed at a high level in the embryo, larva, and adult (Perkins et al. 1990). An Hsc70-4 mutant resulted in a higher lethality rate in the flies during different developmental stages due to the lack of functional endogenous Hsc70 (Elefant and Palter 1999). In the *Drosophila* eye, cytosolic Hsc70-4 cooperates with *auxilin* to regulate Notch signaling by Delta internalization (Hagedorn et al. 2006) and regulates the internalization of Boss ligand in larval eye discs (Chang et al. 2002). From those conclusions, we speculate that higher expression of Unigene0014930 in male ants may relate to visual acuity.

Nguyen et al. (2016) recovered five *Drosophila melanogaster* Hsp70 homologs (CG2918, Hsc70-3, Hsc70-4, Hsc70-5, and Hsp70CB) in Hymenoptera, including in ants, bees, and *Nasonia vitripennis*, with Hsc70-4 leading to two paralogs (h1 and h2) (Nguyen et al. 2016). In our study, in addition to Hsc70-3, Hsc70-4, and Hsc70-5, hypoxia up-regulated protein 1 (HURP1), heat shock 70-kDa protein 9 (Hsp70-9), and heat shock 70-kDa protein 4L (Hsp70-4L) were found in the ants (Table 5), the basal expressions of which were significantly different in the different castes. Usually, Hsc70s act as molecular chaperones of *EcR*/*USP* and are regulated by environmental and physiological stressors (Chichester et al. 2015; Sun et al. 2012). Additionally, Hsc70 can maintain proteostasis by refolding or degrading denatured proteins and preventing aggregation. Yuan et al. (2017) reported that *Litopenaeus vannamei* of Hsc70-5 can tolerate white spot syndrome virus (WSSV) (Yuan et al. 2017). Pan et al. (2014) reported that Hsp70-9 may play an important role in stroke-injury self-repair and PSD-induced injury of hippocampal neurons (Pan et al. 2014). However, the mechanism of different Hsc70 paralogs is not fully distinguished; more experiments are needed to illustrate the crosstalk between Hsp70 family proteins and other developmental genes.

Conclusion

In this study, we have reported the de novo transcriptome of different *P. vicina* ant castes for the first time. These comprehensive sequence resources enrich the genomic platform, and the differential expression analysis will also contribute to better understanding of the gene regulation of caste specificity formation. We found that *PvEcR* and *PvUSP* may function as regulators of caste specificity formation in *P. vicina* adults. Because we did not detect the *PvMet* gene in the sequenced transcriptomes of *P. vicina* adults, we speculated that *PvUSP* may take on JH receptor-like roles in *P. vicina*. In view of the *PvHsp90* and *PvHsc70* richness, we suggest that *PvHsp90* and *PvHsc70*, may be as molecular chaperones of *PvEcR*

and *PvUSP*, may affect adult physiological activities and participate in regulating development, caste differentiation, and caste specificity formation in *P. vicina*, especially the Hsp90 family member Unigene0012302 and the Hsp70 family members Unigene0012706 and Unigene0014930. These results would facilitate the understanding of *P. vicina* development, metamorphosis, and fitness to environmental change.

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