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Short Communication

Comparative transcriptomic analysis provides insights into the response to the benzo(a)pyrene stress in aquatic firefly (*Luciola leii*)

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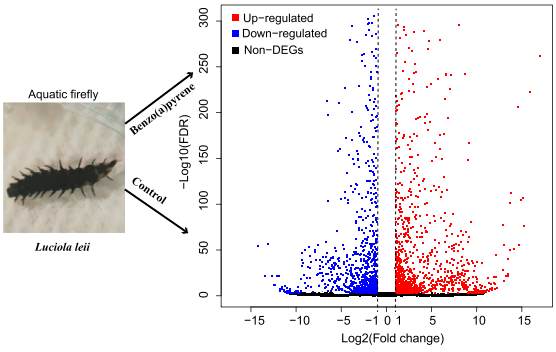
HIGHLIGHTS

- 29,297 of 54,282 genes obtained in *Luciola leii* transcriptome were annotated.
- Most DEGs were involved in xenobiotic biodegradation, biomacromolecule metabolism.
- This study discovered a set of useful SSR and SNP molecular markers.
- Eight DEGs were identified as key BaP-responsive genes.
- This study firstly explored transcriptional response to BaP in aquatic insects.

GRAPHICAL ABSTRACT



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ABSTRACT

Many studies have reported that behavior and bioluminescence of fireflies could be affected by changes in environment conditions. However, little is known about how the deterioration of the aquatic environment affects aquatic fireflies, particularly with respect to molecular responses following exposure to water pollutants, such as benzo(a)pyrene (BaP), which is a key indicator in environmental risk assessment because of the hazards it poses. Here, whole transcriptome sequencing and gene expression analysis were performed on freshwater fireflies (*Luciola leii*) exposed to BaP (concentration of 0.01 mg/L). Four transcriptomic libraries were constructed for the control and treatment groups, including two biological replicates. From the mixed pools (each pool contains 60 individuals from three time points), a total of 54,282 unigenes were assembled. Furthermore, 329,337 of Single-nucleotide Polymorphisms (SNPs) and 1324 of Simple Sequence Repeats (SSRs) were predicted using bioinformatics, which is useful for the future development of molecular markers. Subsequently, 2414 differently expressed genes (DEGs) were identified in response to BaP stress in comparison to the control, including 1350 up-regulated and 1064 down-regulated DEGs. Functional enrichment showed that these DEGs are primarily related to innate immunity; xenobiotic biodegradation and response, biomacromolecule metabolism, biosynthesis, and absorption. Eight key BaP-responsive DEGs were screened to survey the dynamic changes of expression in response to BaP stress at different time points, and to validate the RNA sequencing data using quantitative real-time PCR. The results indicate that the expression of genes encoding UGT, CYP3A, CYP9, CYP6AS5 and ADHP were induced, while those encoding UGT2B10L, PTGDS, and ALDH were reduced, to participate in response to the BaP exposure and potentially help counteract the adverse effects of BaP. This investigation provides insight into the toxicological response of fireflies to the occurrence of water deterioration.

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1. Introduction

The family Lampyridae (Insecta: Coleoptera: Cantharoidae), also known as fire flies or bioluminescent beetles, consists of over 100 genera and 2000 nominated species (Fu, 2005). Fireflies can be divided into terrestrial, aquatic and semi-aquatic lineages according to the living habits of their larvae. Most firefly larvae are terrestrial while a small number of larvae live in freshwater (namely the aquatic fireflies); however, their survival strongly depends on the quality of the water body (Fu et al., 2005). Aquatic fireflies are very rare and are distributed only in Asia and Jamaica. Thus far, only seven species of aquatic fireflies (all of them belonging to the genus *Luciola*) have been described in detail (Fu et al., 2004; Fu et al., 2010). Among these, *Luciola leii* (subfamily Luciolinae) is a widely investigated aquatic species of firefly that has completely aquatic larval stages. *L. leii* is primarily distributed in the middle and lower reaches of the Changjiang River in mainland China, and it is used as an indicator of good water quality (Fu and Ballantyne, 2006; Vongsangnak et al., 2016). However, the mechanism underlying the molecular toxicology in response to stress caused by contaminants in water bodies remains largely unknown. Moreover, the genes and pathways potentially involved in this toxicological and biological response still remain to be identified.

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of hydrocarbons with over 1000 different compounds that are composed of two or more fused benzene rings (Abdel-Shafy and Mansour, 2016). PAHs can enter water bodies through different routes and are found as a mixture of various PAH compounds at differing levels (Xiao et al., 2018). Due to the adverse effects of PAHs on various organisms, including humans, PAHs have become important environmental contaminants of concern. Many investigations demonstrate that PAHs are a crucial driving factor in carcinogenic, mutagenic and teratogenic process (Xiao et al., 2018). Recently, due to ecological deterioration and intensifying human activity, the PAHs can easily be found in rivers, lakes and oceans. Benzo(a)pyrene (BaP) is a PAH known as one of the most potent environmental carcinogens, and human activity is the main cause of its release into the aquatic environment (Xiao et al., 2018). One survey of aquatic sediments in China showed that the BaP content was 0.06–52.12 ng/g in the Yangtze River, 0.19–36.71 ng/g in the Liaohe River, 0.7–69.4 ng/g in Taihu Lake and 18.18–701.12 ng/g in the Pearl River Delta (Jia et al., 2012). The average concentration of BaP in the surface runoff of Hangzhou city, China, was up to 1582 ng/L, and reached 300–400 ng/L in the source water of Meiliang Bay in Taihu Lake (Jia et al., 2012). In addition, few of the currently available studies have investigated the metabolic effects of BaP on freshwater insects (Borchert et al., 1997). However, several recent publications have explored large-scale gene expression changes in response to BaP exposure using RNA sequencing. These studies found that the genes expressed in response to BaP exposure were primarily involved in drug metabolism, xenobiotic metabolism and innate immune signaling in the staghorn coral species *Acropora hyacinthus* embryo and the adult Chinese amphioxus *Branchiostoma belcheri* (Xiao et al., 2018; Zhang et al., 2019a). To date, the molecular mechanisms involving toxicological and biological responses to water contaminants is completely unknown for aquatic insects. Thus, it is necessary to employ suitable insect models, such as the aquatic firefly, to explore molecular toxicological mechanisms in response to important water contaminants. Moreover, the aquatic insect models and their genetic resources will be useful for environmental monitoring and development of technology used for assessing environmental risk in water bodies.

The goal of this study was to explore the transcriptional response of *L. leii* larvae to BaP exposure at a whole-transcriptome level, as well as identify BaP-responsive genes and reveal their functional information. RNA sequencing technology was used to obtain transcriptomes of *L. leii* larvae, and then mass gene expression profiling was analyzed in combination with bioinformatics and quantitative real-time PCR (qRT-PCR). The results will be helpful for understanding the toxicological mechanisms of aquatic insects under water contaminant stress. Furthermore, the genetic resources obtained in this study will be useful for development of novel molecular markers and assessment of environmental risk in water bodies.

2. Materials and methods

2.1. L. leii larvae samples and BaP exposure

Healthy 5th instar *L. leii* larvae (body size: 1.2 ± 0.2 cm) (Fig. S1) were purchased from the Culture Centre of Fireflies, Ganzhou, Jiangxi Province, China, and were maintained at the Evo-dev Institute of Nanjing University, Beihai, Guangxi, China, as previously described (Fu, 2005). The experimental larvae were separated into two jars representing BaP-treated and control groups. A total of 100 individuals were maintained under water in 1.5 L wide-mouth plastic jars containing 1.0 L sterilized lake water. BaP (B1760, Sigma-Aldrich, USA, purity >99%; molecular weight: 252.31) was dissolved into dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA, >99% purity) to reach a stock concentration of 200 μM. Subsequently, the BaP-treated groups were exposed to freshwater containing 0.01 mg/L of BaP. The group treated with an equal concentration (0.03%) of DMSO in freshwater was used as a control. The optimal BaP concentration used for *L. leii* exposure was determined based on previous studies (unpublished data). In brief, the concentration exhibiting the maximum number of BaP-effected-expressed cytochrome P450s (CYP450s), which is a suitable molecular indicator to evaluate BaP pollutants (Camatini et al., 1998; Ryeo-Ok et al., 2013), was selected as the suitable concentration in this study. At 6, 12 and 24 h post-exposure (hpe), 20 *L. leii* larvae individuals were collected from each of the treatment and control groups. The experiments were independently repeated two times, representing two biological replicates. All samples (whole bodies of *L. leii* larvae) were immediately frozen in liquid nitrogen and stored at −80 °C for further RNA extraction.

2.2. RNA isolation, sequencing and transcriptome assembly

Total RNA was extracted from the samples (whole bodies) using the Ambion RNA Pure Kit (Invitrogen, USA). Residual genomic DNA was removed by RNase-free DNase (Qiagen, Germany). An Agilent 2100 Bioanalyzer (Agilent Technologies, USA) was used to confirm RNA structural quality (RNA integrity number (RIN value) ≥ 7). The RNA from 20 individuals that were collected at each time point was diluted to the same concentration (300 ng/μL) with RNase-free water, and these were then pooled equally into the control and treatment samples that were used for further library construction. Library construction was performed at the Beijing Genome Institute (BGI, China) using the Illumina RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocol. The four libraries were sequenced on the Illumina HiSeqTM 2000 platform to obtain 100 bp paired-end reads (BGI, China).

The data filtering and de novo assembly of raw data were implemented under the default parameters unless otherwise stated. First, the raw data was filtered by removing the adaptor sequences, reads with >10% unknown bases (N) and low-quality reads using a FastQC software (v 0.11.7), which was used during quality control to check raw sequence data (Li et al., 2015). Next, de novo assembly of mixed read pooling generated by a combination of the four libraries was performed using Trinity (Grabherr et al., 2011; Haas et al., 2013). The TGICL pipeline was then used to remove redundancy of assembled unigenes by a fast clustering (Pertea et al., 2003). To perform annotation, all unigenes were searched in non-redundancy (NR), gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Swiss-Prot databases using the BLASTX tool (threshold of 1.0e-5). To evaluate the completeness of the assembly, the generated unigenes were compared with a core set of arthropods using BUSCO (Benchmarking Universal Single-Copy Orthologs) v3 (http://busco.

ezlab.org/), which is based on evolutionarily informed expectations of gene content, following previously published methods (Zhang et al., 2017b). The completeness of the unigene set was automatically evaluated, and percentage of "complete", "fragmented", and "missing" were obtained by automatic output of BUSCO software.

2.3. Identification and analysis of SSRs and SNPs

Simple sequence repeats (SSRs), also known as microsatellites, have been widely used as genetic markers, genetic evolution, gene mapping, comparative genomics and population genetics in insects (Wang et al., 2009). In brief, MIcro SAtellite (MISA) (v1.0) software was used to detect SSRs in all the assembled unigenes (Thiel et al., 2003). The SSR primer design was then performed using the Primer3 tool (Triinu and Maido, 2007). Single nucleotide polymorphisms (SNPs) are single nucleotide mutations that occur between samples or individuals at the DNA or RNA level. Because of variations in some key functional nucleotide mutations, SNPs may lead to changes or losses of protein function, which can result in adaptive evolution of insects to their habitats (Jha et al., 2016). Therefore, SNPs are useful molecular markers during investigations of environmental adaptation using aquatic fireflies. Alignment of multiple clean reads corresponding to unigenes was conducted using HISAT (v0.1.6-beta) (Daehwan et al., 2015) and detection and filtering of low-quality SNPs was conducted using GATK analysis (v3.4-0) (McKenna et al., 2010).

2.4. Analysis of differentially expressed genes

To quantify gene expression levels, clean reads of each library were first mapped to the reference unigene set of the *L. leii* transcriptome assembled in this study using Bowtie 2 under the default parameters (Langmead et al., 2009). Furthermore, the RSEM (RNA-Seq by Expectation Maximization) tool was used to calculate the fragments per kilobase of unigenes per million fragments mapped (FPKM) (Zhang et al., 2017d) according to the amount of mapping reads on each unigene. Sample reproducibility was assessed between the two biological replicates based on the FPKM values of each unigene. Differentially expressed genes (DEGs) were determined and extracted using DESeq software, which is an R package used for identification of DEGs for transcriptomes containing biological replicates (Anders and Huber, 2010). The DEGs identified by the DESeq tool shows more conservative and lower false rates than those identified by other software with similar function (e.g. DEGseq and NOISeq) (Behringer et al., 2015). The threshold for DEG determination was set as fold changes (FC) ≥ 2 (|log2 ratio| ≥ 1) and 0.01 *p*-values (Wald test in DESeq) corrected by the Benjamini-Hochberg method (false discovery rate, FDR).

2.5. Functional enrichment analysis of differentially expressed genes

To understand the main biological, cellular and molecular functions, as well as the signaling pathways of DEGs, the corresponding GO and KEGG annotations were extracted for each annotated DEG using custom Perl scripts. Next, GO enrichment analysis of DEGs was performed using the Blast2GO pipeline (Conesa et al., 2005) with Fisher's exact test (*p*-value calculation), and they were enriched into three subcategories, namely biological processes, molecular function, and cellular components. Next, KOBAS 2.0 was used with the default options (http://kobas.cbi.pku.edu.cn/) to conduct KEGG enrichment analysis for the DEGs. The Benjamini-Hochberg adjustment was used for correction of the significant level of all the GO and KEGG terms. The FDR threshold was set as 0.05.

2.6. Validation of RNA-Seq data and observation of gene expression by qRT-PCR

To validate the RNA-Seq data, 12 DEGs (eight key BaP-responsive and four randomly selected DEGs) were used for qRT-PCR analysis from a sample of the same RNA that was used for the RNA-Seq experiments. Furthermore, the expression level of eight key BaP-responsive DEGs were detected in a replicated experiment using 5th instar *L. Leii* larvae exposed to BaP for 6, 12, 24 and 48 h (hpe). The sampling and RNA purification was conducted following methods described in Sections 2.1 and 2.2. Specific qRT-PCR primers were designed using Beacon Designer 7 (Tables S1). Synthesis of the first-strand cDNA and qRT-PCR analysis was performed according to methods used in our previous studies (Zhang et al., 2017e). The elongation factor 1A (*EF1A*) gene was used as a housekeeping gene (Tang et al., 2017) and each qRT-PCR reaction was conducted with three technical replicates and two biological replicates. Data was visualized with the SDS tool (version 2.0) in the ABI 7300 Real-Time PCR system. Gene expression normalization and statistics were conducted based on the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Namely, Ct values of all technical replicates of each gene in each sample were generated by PCR system. Then, in each biological replicate, mean value of Ct, $\triangle$Ct, $\triangle\triangle$Ct and $2^{-\triangle\triangle Ct}$ values of each gene were calculated. Next, mean and standard deviation (SD) of $2^{-\triangle\triangle Ct}$ values from two biological replicates were obtained. The significance of differences between control and BaP-treated firefly at each time point (6, 12, 24 and 48 hpe) was evaluated with one-way ANOVA followed by Bonferroni post-tests using IBM SPSS statistics 22.

3. Results and discussion

3.1. Overview of RNA sequencing data

A flow diagram of the experiments and bioinformatic analyses is presented in Fig. 1A. After RNA sequencing and quality control, approximately 3 Gb of clean reads were generated from each of the four libraries, and the Q20 percentage of the clean reads in each library was higher than 97.45% (Table 1). A total of 294,391,102 clean reads, with an average GC content of 41.24%, were generated from the four libraries (Table 2). These clean reads have been submitted to the Sequence Read Archive (SRA) database of NCBI (Accession numbers SRR8145632-SRR8145635). A total of 54,282 unigenes and 105,644 transcripts was obtained, with an average unigene length of 999 bp and an N50 of 2409 bp. Functional annotation analysis showed that 53.97% of the unigenes (29,297) were annotated to at least one database of NR, Swiss-Prot, GO and KEGG, while the remaining unigenes were not annotated. The length distribution showed that 16.43% of the unigenes were >2000 bp (Fig. S2). Overall, the large-scale mixed assembly of the four libraries generated a higher quality transcriptome than that had been previously obtained by other studies of aquatic fireflies (*Luciola aquatilis*) (Vongsangnak et al., 2016). For example, the N50 and mean values of unigene length in this study (2409 bp and 999 bp, respectively) are longer than that (1889 bp and 978 bp, respectively) of Vongsangnak et al. (2016); moreover, in this study (16.43%), the percentage of unigenes with >2000 bp is higher than that in previous publications (13.58%) (Vongsangnak et al., 2016). In addition, results of the BUSCO analysis based on 2675 near-universal single-copy orthologs in the arthropod gene set showed that for the *L. leii* transcriptome assembled in this study, 68.4% of the unigenes were "complete", 16.2% were "fragmented" and the remaining genes were "missing". In comparison to the 46 other arthropod species assemblies (Theissinger et al., 2016), the results show that the integrity of the assembled unigenes is higher than that of the majority of assembled transcriptomes. Therefore, a high-quality unigene set for *L. leii* larvae was obtained here and subsequently used for downstream analyses. The high quality unigene set for *L. leii* larvae is a reasonable and novel outcome of the present work.

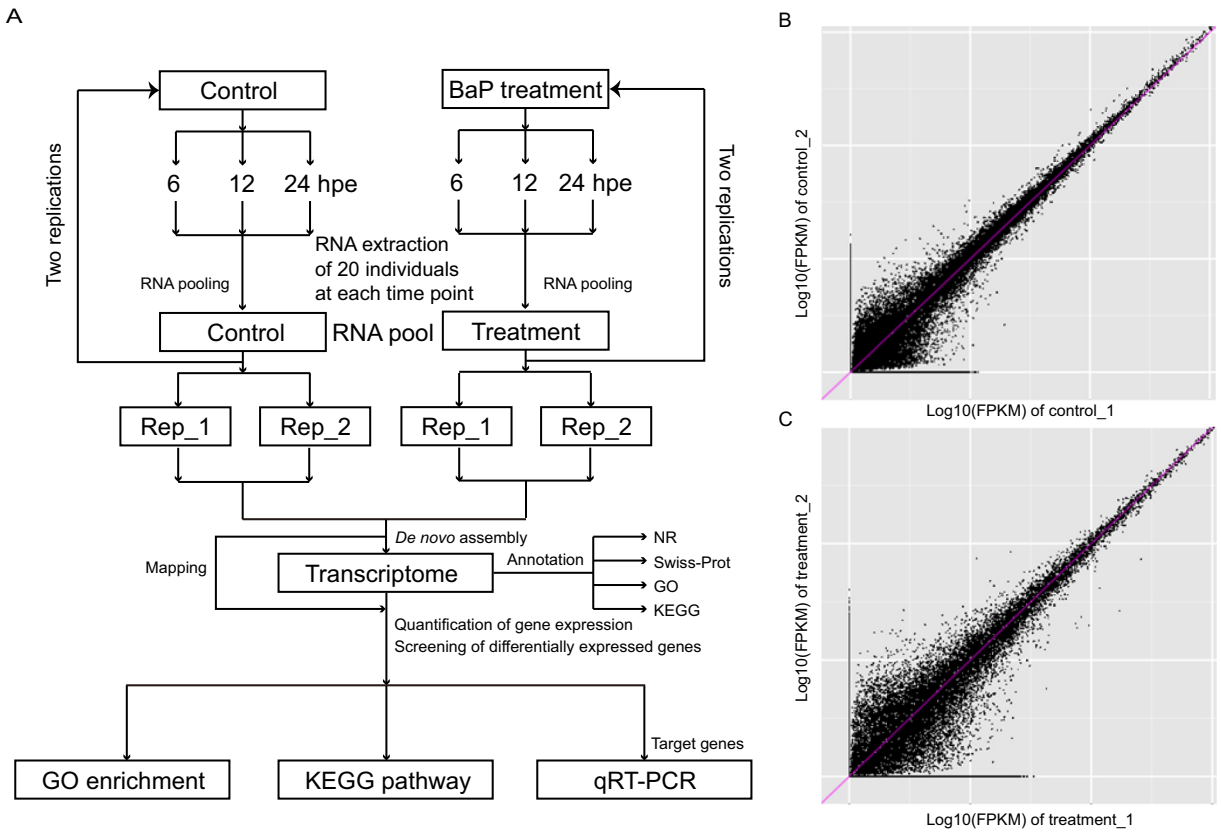

Fig. 1. (A) A flow chart of experiments and bioinformatic analyses. Hpe indicates hours post-exposure. (B) Correlation analysis between two control samples based on gene expression levels. (C) Correlation analysis between two treatment samples based on gene expression levels. When black dots are close to the pink oblique line, this indicates that the two samples are highly similar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. SSRs and SNPs identification

In total, 1342 SSR markers were identified in 1278 unigenes. Among these unigenes, 54 contained >1 SSR. Among those SSRs, the most frequent motif type was tri-nucleotide (642, 47.84%), followed by di-nucleotide (400, 29.81%), mono-nucleotide (255, 19.00%), quad-nucleotide (26, 1.94%) and other (19, 1.42%) nucleotide motif types (Fig. 2A). Moreover, the size of the SSR sequences was mainly distributed in the 12–24 bp range, occupying 99.25% of the total SSRs. Among the SSRs, a total of 29 motif types were detected. The repeated AAT/ATT nucleotide type was the most frequent motif type in this investigation (286, 21.31%), followed by A/T (253, 18.85%) and AT/AT (238, 17.73%). In the current investigation, a total of 329,337SNP sites were detected. The number of transition and transversion types was 221,500 and 107,837, respectively (Fig. 2B). The frequency of SNPs in the *L. leii* larvae transcriptome was 1/165 bp, meaning that on average an SNP site could be found per 165 bp of unigene length. The number of A/G and C/T was maximized among all six SNP types, accounting for 33.70% and 33.56%, respectively, while the other four SNP types (A/C, A/T, C/G and G/T) represented 7.54%, 12.74%, 4.93% and 7.53%, respectively. This study presents the first detection of molecular markers for aquatic fireflies. Genetic diversity evaluation based on SSR and SNPs is an effective approach for conservation biology research of aquatic animals (Miller et al., 2014). Therefore, these molecular markers are valuable for the protection of *L. leii* larvae in water environments.

3.3. Analysis of differentially expressed genes

Clean reads were mapped to the reference unigene set for each library and the mapping statistics are summarized in Table 1. The percentage of total mapped reads ranged from 83.46% to 88.51% for the four libraries, of which approximately 65% and 22% accounted for unique and multi-position matches, respectively, for each library. A

Table 1
Summary of RNA-Seq information for each sample.

Category	Control		BaP treatment	
	C_1	C_2	T_1	T_2
Total clean reads	75,866,242	71,280,880	74,922,326	72,321,654
Total mapped reads (%)	88.33	88.51	83.46	83.61
Unique match (%)	64.90	63.99	67.47	65.16
Multi-position match (%)	23.43	23.62	20.99	22.37
Total unmapped reads (%)	11.67	12.39	11.54	12.47
Perfect match (%)	48.54	48.36	47.81	48.07
Q20 percentage	97.51	97.53	97.47	97.51

Table 2
Summary of the *Luciola leii* transcriptome generated with Trinity assembler software.

Item	Number
Number of clean reads	294,391,102
Total transcripts	105,644
Total unigene length(nt)	54,231,639
Total unigene number	54,282
GC content (%)	41.24%
Unigene N50 (nt)	2409
Mean unigene length (nt)	999
NR annotation	28,562
Swiss-Prot annotation	22,156
GO annotation	14,047
KEGG annotation	20,511
All annotated unigenes	29,297

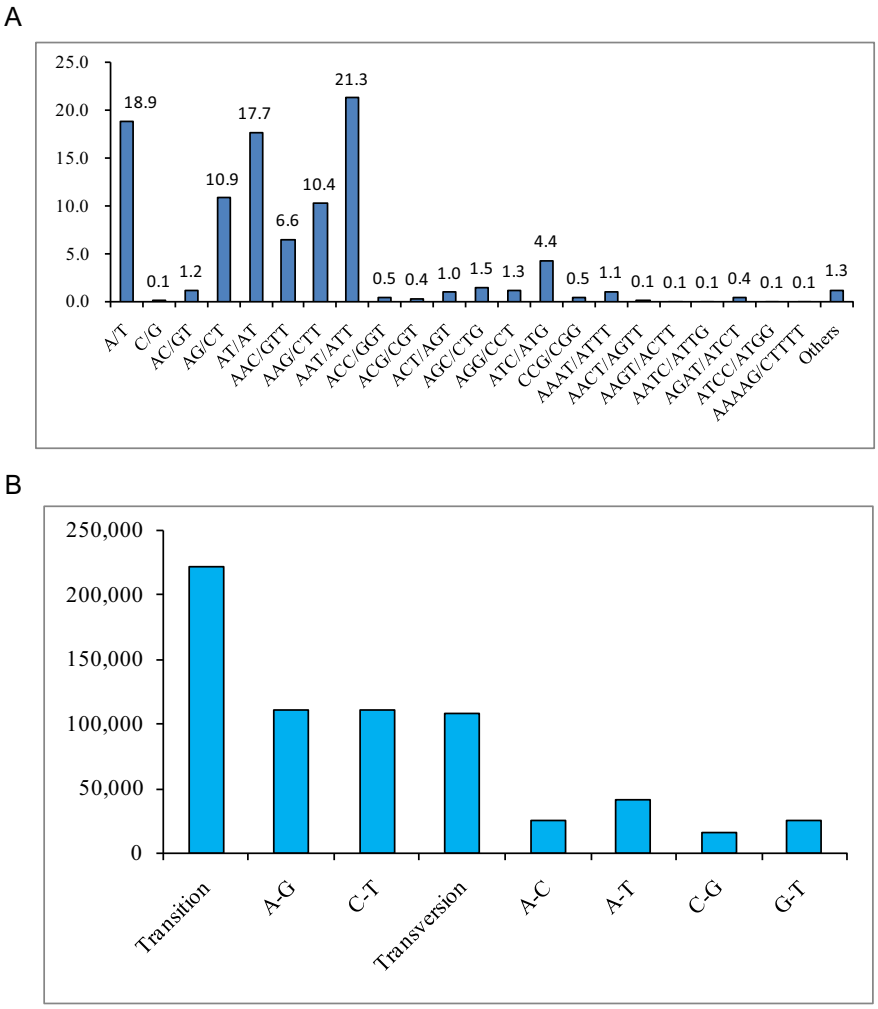

Fig. 2. (A) Frequency of classified repeat SSR types. (B) SNP variation types of *L. leii* larvae.

high mapping rate of clean reads indicates the results of RNA sequencing were robust and can be used for further quantification of gene expression. Sample repeatability between two replicates was assessed by calculating Pearson's correlation coefficient based on the FPKM values of the unigenes. The Pearson's correlation coefficients were >0.90 between the two biological replicates from treatment or control samples (Fig. 1B and C). A high correlation between biological replicates indicates that an effective experimental treatment was obtained and that accidental error in a single sample was effectively avoided in this study.

A total of 2414 DEGs were identified in response to BaP stress, of which expression of 1350 DEGs were up-regulated, while 1064 DEGs were down-regulated in the *L. leii* larvae treated by BaP in comparison to control (Table S2). According to log2 FC in treatment compared to the control, the top 15 up- and down-regulated DEGs are presented in Table 3. The gene encoding mitochondrial arginyl tRNA synthetase (RARS) showed the most up-regulated expression of the DEGs, followed by cuticle protein 8 (CP8) and cytochrome P450 monooxygenase CYP304E2 (CYP304E2). Conversely, the gene encoding cAMP-binding protein 1 (CAPA1) was the most up-regulated gene, followed by the gene encoding autophagy-related protein 8 (ATG8) and chymotrypsin-1 (CHYM1). RARS is a gene that participates in protein translation and hypoxia response. Previous research has demonstrated that functional changes of RARS alters the survival rate of *Caenorhabditis elegans* under hypoxic stress and that the tolerance level to hypoxia is inversely correlated with the translation rate (Anderson et al., 2009). Furthermore, BaP exposure induced rapid accumulation of reactive oxygen species (ROS) in fish liver, which significantly enhanced hypoxia-related gene expression (Yu et al., 2008). Mitochondria, an energy production and metabolic factory, play the most important role in hypoxia resistance of insects (Zhang et al., 2017c). In the list of the top 15 DEGs, several DEGs were detected that involved mitochondrial function, such as genes encoding superoxide-generating NADPH oxidase heavy chain subunit C (NOXC), cytochrome *c* oxidase subunit 5A, mitochondrial (COX5A) and cytochrome-c1-heme lyase (CYT2). Desouki et al. (2005) reported that NOX could generated ROS in human cells and revealed that knockdown of mitochondrial genes caused down-regulation of gene encoding NOXs while expression of NOX genes was rescued by complementation with wild type mitochondrial genes. Previous studies reported that the oxidative stress caused by BaP disturbed the expression of COX genes in earthworms (*Eisenia fetida*) (Lin et al., 2008). Moreover, changes in expression of COX genes are sensitive, and these genes have been demonstrated as an oxygen sensor in brain neurons (Susann et al., 2010). Cytochromec1, which is a subunit of the mitochondrial ubiquinol-cytochrome-c reductase, must be connected with heme protein under CYT2 catalysis during cytochrome *c*1 import into the mitochondria (Nicholson et al., 1989). Thus, we speculated that ROS generation caused by BaP stress leads to differential expression of these four function- and hypoxia-related mitochondrial genes (RARS, NOXC, COX5A, CYT2) between BaP-treated and control groups. Furthermore, they may be potential biomarkers for oxidative stress following xenobiotic contaminant exposure in aquatic *L. leii*. CYPs have been extensively studied in insects, with many investigations focusing on the gene function of CYP in aquatic animals in response to BaP challenge.

For example, Tian et al. (2014) demonstrated that mollusk CYPs can be regulated by marine contaminants and the expression suppression of several CYP-like genes may be caused by the high toxic effect and oxidative damage of BaP in *Chlamys farreri*. In addition, studies show that BaP suppression of immune system function relies on CYP-catalyzed processes of immunotoxic metabolism in Japanese medaka (*Oryzias latipes*) (Pannetier et al., 2019). Furthermore, CYPs have been widely considered as a set of suitable molecular indicators for BaP pollution (Camatini et al., 1998; Ryeo-Ok et al., 2013). In this study, expression of genes encoding cytochrome P450 monooxygenase CYP304E2 (CYP304E2), cytochrome P450 6BQ13 (CYP6BQ13) and cytochrome P450 345B1 (CYP345B1) was altered by BaP exposure. Thus, our investigation suggests that BaP stress influences the detoxification function of *L. leii* and also indicates that these CYPs may be useful as molecular toxicological indicators of aquatic fireflies in water environments.

Interestingly, insect cuticle is the main component of the body wall and functions by maintaining the morphology of the body, inhibiting evaporation of body water and resisting invasion of xenobiotics (Vincent and Wegst, 2004). Loss or reduction of cuticle protein content leads to abnormal or even loss of cuticular function, thus affecting normal insect growth and development (Moussian et al., 2006). Therefore, differential expression of the genes encoding cuticle protein 8 (CP8) and cuticle protein 7 (CP7) suggest that BaP exposure probably affects the developmental processes of *L. leii* larvae. Furthermore, we found upregulation of genes involved in ubiquitin and cell death, such as those encoding ubiquitin-conjugating enzyme E2G (UBE2G), ubiquitin-like protein 5 (UBL5) and cell death protein 2 (CED2) under BaP stress. Song et al. (2019) found that various doses of BaP significantly affected the expression of these related genes when using RNA sequencing to study polar cod (*Boreogadus saida*). In addition, many studies have found that heat shock proteins (HSPs) could protect against DNA damage caused by BaP (Gong et al., 2007; Zheng et al., 2008). In this study, several of the top 15 DEGs were related to the protection of DNA and repair of DNA damage, such as hsp70/hsp90 organizing protein-like protein (HS718), heat shock protein 100 (HSP100) and DNA mismatch repair protein Mlh1 (MLH1). This indicates that prevention of DNA damage caused by BaP is a key biological process in the response of *L. leii* to BaP stress. Additionally, we also identified two genes encoding cytosolic carboxypeptidase 1 (AGTPBP1) and luciferin 4-monooxygenase (LUCI). Roles of AGTPBP1 and LUCI are not well discussed due to complexity and diversity of toxicological response, but these two genes were identified as DEGs after BaP treatment in this study, thereby showing the potential impact of BaP on their biological function. The genes without annotated information are presented as hypothetical proteins, and their function should be further analyzed.

3.4. Functional enrichment analysis of differentially expressed genes

Following GO functional enrichment analysis of the DEGs, 46 biological process terms were identified that involved the primarily immune response, response to stimulus, nucleic acid metabolism and repair, morphogenesis and development, and amino acid metabolic processes, such as response to stimuli, immune response, DNA metabolism and the cuticle chitin metabolic process (Table S3). For the eight enriched GO terms associated with molecular function, we found that these categories were primarily related to biomacromolecule binding and structural constituents of extracellular matrices and cuticles, such as sequence-specific DNA binding, structural constituent of cuticle, extracellular matrix structural constituent. Additionally, for the 13 cellular component terms enriched by DEGs, those involving the extracellular region and complex and nucleus function were overrepresented. In addition, 32 KEGG pathways enriched by the DEGs were obtained, indicating that the gene expression of pathway members was altered under BaP stress (Table S4) and that these pathways are key signaling transduces of a progressive molecular response to BaP exposure in *L. leii* larvae. The significantly enriched KEGG terms were primarily associated with xenobiotic and drug metabolism, disease, immune response, substance digestion and absorption, such as protein digestion and absorption,

Table 3
Top 15 up- and down-regulated genes between the control and treatment groups.

Gene ID	log2Ratio (treatment/control)	FDR	Annotation	Gene name
The top 15 most up-regulated genes				
Unigene26775	17.04	7.37E-263	Mitochondrial arginyl tRNA synthetase	RARS
Unigene10953	15.87	1.39E-223	Cuticle protein 8	CP8
Unigene11072	15.25	2.81E-77	Hypothetical protein	/
Unigene10015	15.18	1.04E-107	Cytochrome P450 monooxygenase CYP304E2	CYP304E2
Unigene11983	14.93	3.36E-105	Cytochrome P450 6BQ13	CYP6BQ13
Unigene33539	14.72	2.76E-56	Ubiquitin-conjugating enzyme E2G	UBE2G
Unigene10425	14.59	1.27E-206	Hsp70/Hsp90 organizing protein-like protein	HS718
Unigene8226	14.14	3.25E-52	Hypothetical protein	/
Unigene11616	13.81	4.85E-113	Hypothetical protein	/
Unigene8917	13.71	9.50E-102	Cuticle protein 7	CP7
Unigene17694	13.71	2.47E-50	Cytochrome P450 345B1	CYP345B1
Unigene19571	13.50	2.18E-24	Superoxide-generating NADPH oxidase heavy chain subunit C	NOXC
Unigene37979	13.48	2.17E-24	Hypothetical protein	/
Unigene29414	13.46	6.78E-22	Ubiquitin-like protein 5	UBL5
Unigene9415	13.44	6.01E-41	Cell death protein 2	CED2
The top 15 most down-regulated genes				
Unigene27586	−16.94	0	Hypothetical protein	/
Unigene30398	−14.22	3.33E-55	cAMP-binding protein 1	CAPA1
Unigene18974	−13.45	1.44E-22	Autophagy-related protein 8	ATG8
Unigene12583	−13.08	8.76E-58	Chymotrypsin-1	CHYM1
Unigene12275	−12.64	1.44E-22	Cytochrome b5	CYTB5
Unigene31925	−12.44	6.73E-13	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A
Unigene2849	−12.30	4.75E-12	Hypothetical protein	/
Unigene20059	−12.15	5.34E-22	Cytosolic carboxypeptidase 1	AGTPBP1
Unigene11399	−11.87	5.34E-22	DNA mismatch repair protein Mlh1	MLH1
Unigene24204	−11.78	1.14E-08	Hypothetical protein	/
Unigene6022	−11.75	1.95E-06	Luciferin 4-monooxygenase	LUCI
Unigene6510	−11.54	8.56E-10	Cytochrome c1, heme lyase, mitochondrial	CYT2
Unigene7788	−11.51	7.90E-08	Heat shock protein 100	HSP100
Unigene36211	−11.35	1.32E-05	Hypothetical protein	/
Unigene30489	−11.34	1.03E-06	Hypothetical protein	/

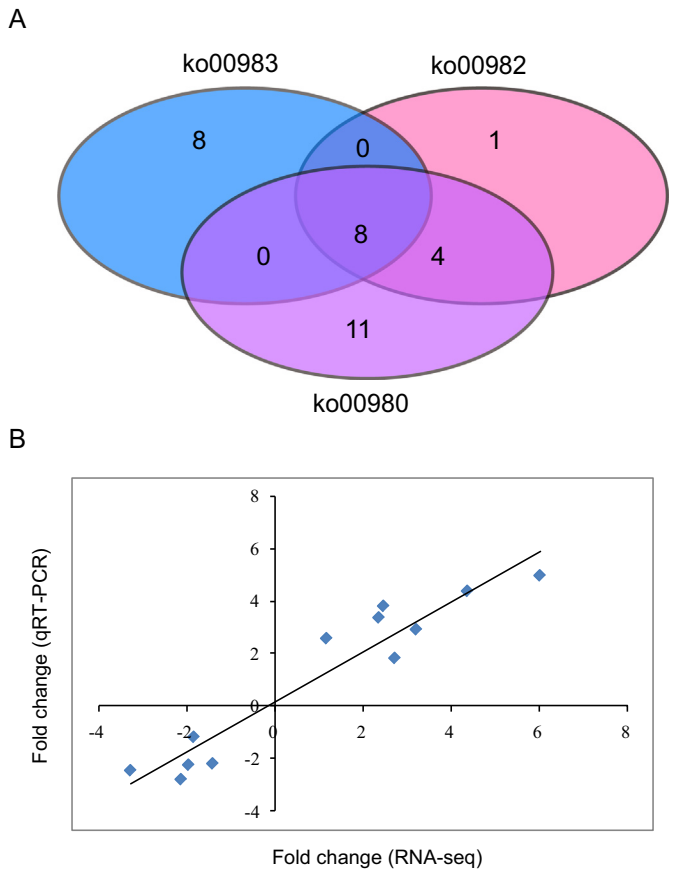

Fig. 3. Venn diagram of DEGs belonging to three KEGG pathways involving xenobiotic biodegradation and response. (D) Correlation between the relative fold changes in expression from RNA-Seq and qRT-PCR analyses. Three technical replicates were conducted for each of the two biological replicates.

drug metabolism - other enzymes, drug metabolism - cytochrome P450, metabolism of xenobiotics by cytochrome P450, tyrosine metabolism, Toll-like receptor signaling pathway, *Vibrio cholerae* infection and malaria. Most of the signaling pathways identified in the current study were also found in response to BaP stress in other animals, including marine cephalochordate-amphioxus (*Branchiostoma belcheri*) (Zhang et al., 2019a), coelenterate-coral (*Acropora hyacinthus*) embryo (Xiao et al., 2018), teleostean polar cod (*B. saida*) (Song et al., 2019) and annulata-earthworm (*Eisenia fetida*) (Zhang et al., 2017a). This analysis indicates that, to a great extent, the aquatic firefly shares similar molecular mechanisms regarding the BaP response with other animal groups.

3.5. Analysis of key BaP-responsive genes

Three well-documented KEGG terms involving xenobiotic biodegradation and metabolism were significantly enriched, including metabolism of xenobiotics by cytochrome P450, drug metabolism-cytochrome P450 and drug metabolism-other enzymes (Guo et al., 2013; Guo et al., 2016). A total of eight DEGs were shared among these three pathways (Fig. 3A), suggesting a key role of these eight DEGs in xenobiotic biodegradation. These genes were annotated to the CYP members, glucuronosyltransferase (UGT), UDP-glucuronosyltransferase 2B10-like (UGT2B10L), prostaglandin-H2 D-isomerase (PTGDS), alcohol dehydrogenase, propanol-preferring (ADHP) and aldehyde dehydrogenase (NAD(P)+) (ALDH). These gene family/genes encode several proteins that can metabolize a wide variety of aquatic xenobiotic contaminants, such as nitrite, farm chemicals, organic compounds, heavy metal, plant-derived toxic substance and the products of oxidative stress (i.e. free radicals and ROSs) (Guo et al., 2013; Guo et al., 2016; Zhang et al., 2019b). Thus, they are identified as key BaP-responsive genes (Gene set 1, GS1) in the current study. In addition, to conduct an effective qRT-PCR analysis to validate the results of RNA sequencing, four additional DEGs were randomly selected for further analysis. Results of qRT-PCR analysis of the 12 DEGs showed a close correlation (Pearson correlation coefficient = 0.912, $p < 0.01$) of fold change in expression between the RNA sequencing and qRT-PCR results, thereby supporting the accuracy of the RNA sequencing and analysis in this experiment (Fig. 3B).

Expression changes of the GS1 genes in the BaP treatment in comparison to the control are presented in detail in Fig. 4 for multiple time points. The results show that the expression levels of UGT, CYP3A, CYP9 and CYP6AS5 were significantly induced at all time points BaP-post-exposure (6, 12, 24 and 48). Meanwhile, the gene encoding UGT2B10L showed significant down-regulation of expression at 6, 12 and 24 hpe. Furthermore, previous investigations reported that these

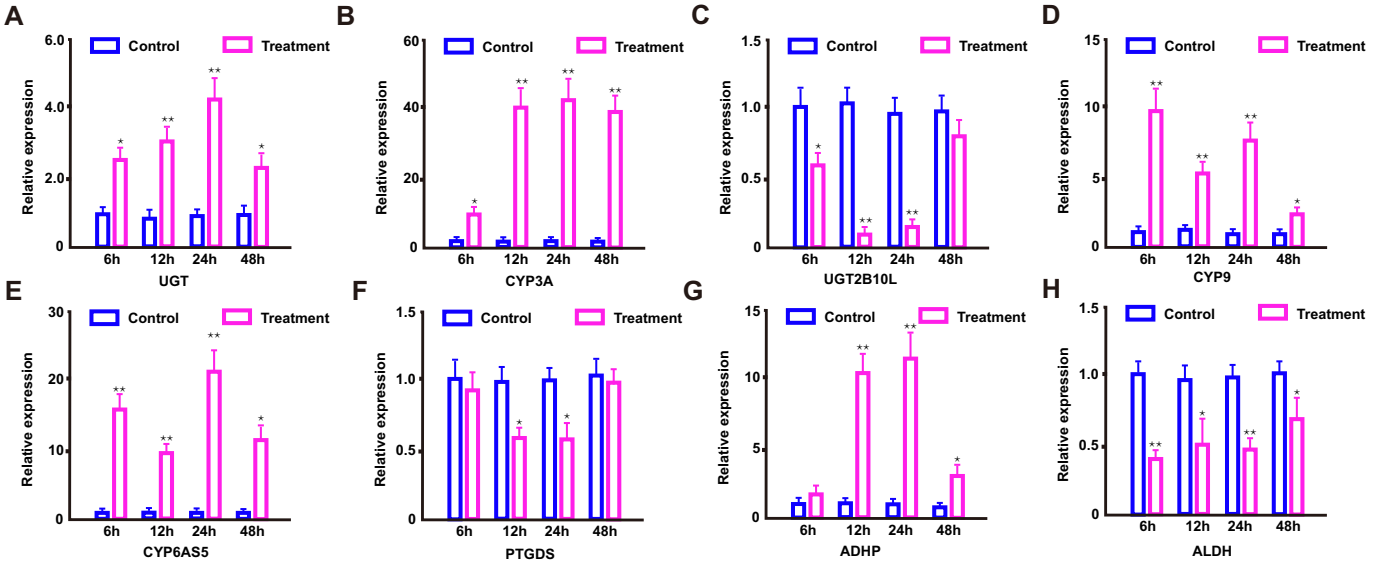

Fig. 4. qRT-PCR results of DEGs shared by the three xenobiotic biodegradation and metabolism pathways in *L. leii larvae* exposed to BaP stress at different time points. (A) UGT, (B) CYP3A, (C) UGT2B10L, (D) CYP9, (E) CYP6AS5, (F) PTGDS, (G) ADHP and (H) ALDH. Data are presented as the mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the corresponding time points of the control; one-way ANOVA plus Bonferroni post-tests.

detoxifying proteins (CYPs and UGTs) participated in the response to oxidative stress in rat astrocytes (Gradinaru et al., 2012). The gene encoding alcohol dehydrogenase, propanol-preferring (ADHP) was significantly up-regulated at 12, 24 and 48 hpe. A high expression of ADH genes was identified as important to the growth of *Metarhizium acridum* under hypoxia exposure by detoxification of acetaldehyde (Nora et al., 2011). In addition, the expression of PTGDS and ALDH genes were down-regulated at different time points following BaP exposure. Several studies have reported that the expression level of ALDH can be altered by BaP stress, and ALDH is widely considered as a common marker of cancer stem cells due to its significant effect on the expression levels of stem cell markers in human BEAS-2B cells (Liu et al., 2016). These findings indicate that oxidative stress caused by BaP exposure is a crucial factor for triggering the metabolic and toxicological response of *L. leii* larvae to xenobiotics in the water environment.

4. Conclusions

For the first time, the toxicological response of aquatic insects to contaminants was explored using RNA sequencing. The transcriptome of the aquatic firefly *L. leii* was generated based on large-scale sequencing data. A number of molecular markers (SSRs and SNPs) that can be used in investigations of genetic diversity were predicted, and DEGs involving BaP stress were also identified. These DEGs are primarily involved in diverse signaling pathways related to disease, xenobiotic biodegradation, immune response and prevention of DNA damage. The expression levels of eight key BaP-responsive genes, which were newly identified in this study, were further investigated using qRT-PCR, thereby confirming their crucial roles in response to BaP exposure in *L. leii*. This study provides valuable information to further elucidate the molecular response of aquatic insects following exposure to various environmental stresses and indicates a complex and diverse network of molecular toxicology in *L. leii*. However, due to the majority of unknown factors related to the toxicological response to BaP exposure, several obtained results that cannot be reasonably discussed require further exploration.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.156.

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