Received: 29 July 2019 | Revised: 17 September 2019 | Accepted: 19 September 2019

DOI: 10.1002/arch.21633

RESEARCH ARTICLE

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

Identification and characterization of miRNAs in an endoparasitoid wasp, *Pteromalus puparum*

Shan Xiao[1] | Beibei Wang[1] | Kai Li[2] | Shijiao Xiong[1] | Xinhai Ye[1] | Jiale Wang[1] | Jiao Zhang[1] | Zhichao Yan[1] | Fang Wang[1] | Qisheng Song[3] | David W. Stanley[4] | Gongyin Ye[1] | Qi Fang[1]

[1]State Key Laboratory of Rice Biology & Ministry of Agriculture and Rural Affairs Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou, China

[2]Institute of Biological Sciences and Biotechnology, Donghua University, Shanghai, China

[3]Division of Plant Sciences, College of Agriculture, Food and Natural Resources, University of Missouri, Columbia, Missouri

[4]USDA Agricultural Research Service, Biological Control of Insects Research Laboratory, Columbia, Missouri

Correspondence
Qi Fang, Institute of Insect Sciences, Zhejiang University, Yuhangtang Road 866, Hangzhou 310058, China.
Email: fangqi@zju.edu.cn

Funding information
The National Key R&D Program of China, Grant/Award Number: 2017YFD0200400; The Natural Science Foundation of Zhejiang Province of China, Grant/Award Number: LY18C140001; The Key Program of National Natural Science Foundation of China, Grant/Award Number: 31830074; The Program for Chinese Innovation Team in Key Areas of Science and Technology of the Ministry of Science and Technology of the People's Republic of China, Grant/Award Number: 2016RA4008; The Program for Chinese Outstanding Talents in Agricultural Scientific Research of the Ministry of Agriculture and Rural Affairs of the People's Republic of

Abstract

MicroRNAs (miRNAs) are a form of endogenous small noncoding RNAs that regulate protein-coding gene expression at the posttranscriptional level. So far, knowledge of miRNAs in parasitoids remains rudimentary. We investigated miRNAs in *Pteromalus puparum*, a pupal endoparasitoid wasp with genome and transcriptome sequences completed. In this study, we constructed eight small RNA libraries from selected developmental stages and genders: male embryos, male larvae, male pupae, male adults, mixed-sex embryos, mixed-sex larvae, mixed-sex pupae, and female adults. We identified 254 mature miRNAs with 5p/3p arm features originated from 75 known and 119 novel miRNA genes in *P. puparum*, 88 of which reside in 26 clusters. The miRNAs in more than half of the clusters exhibit a consistent expression pattern, indicating they were co-transcribed from a long transcript. Comparing miRNA expression in the eight libraries, we found that 84 mature miRNAs were differentially expressed between embryos and larvae, 20 between larvae and pupae, and 26 between pupae and adults. We found some miRNAs were differentially expressed between sexes in embryos (10), larvae (29), pupae (8), and adults (14). Target predictions resulted in 211,571 miRNA–mRNA interactions for 254 different mature

China; The Major International (Regional) Joint Research Project of NSFC, Grant/Award Number: 31620103915

miRNAs. These miRNAs may be involved in sexual and developmental regulation of gene expression.

KEYWORDS
development, miRNA, *Pteromalus puparum*, sex

1 | INTRODUCTION

Parasitoid wasps are ideal hymenopteran study models due to their short life history, high fecundity, little inbreeding depression effects and haplodiploidy sex determination system (Werren & Loehlin, 2009). *Pteromalus puparum* is a pupal endoparasitoid wasp that parasitizes some butterflies including the global vegetable pest, the cabbage white butterfly, *Pieris rapae* (Cai, Ye, & Hu, 2004). The female wasps inject venom without polydnaviruses into host pupae to make a suitable environment for their offspring (Cai et al., 2004; Zhu, Ye, Dong, Fang, & Hu, 2009). So far, 70 candidate venom proteins have been identified (Yan et al., 2016). The relative simple venom composition and available genomic and transcriptomic data facilitates our investigation of this host-endoparasitoid system.

MiRNAs are endogenous noncoding RNAs that efficiently fine tune protein-coding gene expression at the posttranscriptional level through typically binding to the 3′-untranslated region (3′-UTR) of target genes (Bartel, 2004). miRNAs are involved in diverse biological processes such as development (Reinhart et al., 2000), aging (N. Liu et al., 2012), metabolism (Varghese, Lim, & Cohen, 2010), immune responses (O'Connell, Rao, Chaudhuri, & Baltimore, 2010), reproduction (Y. Zhang et al., 2016), and circadian rhythms (Chen et al., 2014). Although the insect miRNAs are understudied, the significance of miRNAs can be taken from their actions in human medicine. For example, dysregulation of miRNAs has been implicated in cancers and many other human diseases (Iliopoulos, Hirsch, & Struhl, 2009; Mendell, 2008; Rupaimoole, Calin, Lopez-Berestein, & Sood, 2016), and miRNAs are emerging as novel targets for therapeutic approaches but also as potent diagnostic biomarkers due to their cell-type specificity (Mendell, 2008; Roncarati et al., 2014).

MiRNAs participate in various insect physiological processes such as insect metamorphosis. Let-7-complex miRNAs are implicated in metamorphosis in species such as the fruit fly, *Drosophila melanogaster* (Caygill & Johnston, 2008), the silkworm, *Bombyx mori* (Ling et al., 2014), and the cockroach, *Blattella germanica* (Belles, 2017; Rubio & Belles, 2013). *D. melanogaster* lacking let-7 and miR-125 have wing defects and delayed maturation of neuromuscular junctions during metamorphosis (Caygill & Johnston, 2008). *miR*-2 family miRNAs are crucial for *B. germanica* nymphs molting to normal adults by decreasing *Kr-h1* mRNA levels (Lozano, Montanez, & Belles, 2015). miRNAs act in innate immunity in insects. Overexpression of miR-34 in *D. melanogaster* improves the survival of gram-negative bacterial infection (Xiong et al., 2016). miR-8 negatively modulates drosomycin expression by targeting the genes *Dorsal* and *Ush* in *D. melanogaster* (Lee & Hyun, 2014). In *Plutella xylostella*, downregulation of miR-8 after parasitization leads to significant declines in *Serpin27* and subsequent upregulation of the antibacterial protein gloverin (Etebari & Asgari, 2013). miRNAs also act in insect reproduction. In *Aedes aegypti*, miR-277 regulates lipid metabolism and reproduction by interacting with two insulin/FOXO pathway genes, insulin-like peptides 7 and 8. CRISPR-Cas9-mediated miR-277 depletion leads to severe defects in primary follicle formation (Ling, Kokoza, Zhang, Aksoy, & Raikhel, 2017), and miR-309 promotes ovarian follicle formation by targeting SIX homeobox 4 (Y. Zhang et al., 2016).

Studies of hymenopteran miRNAs were mainly carried out on honey bee, *Apis mellifera*. Analysis of scores of small RNA libraries reveals miRNA genes in *A. mellifera*, of which 254 have been deposited in the miRBase 21 (www.mirbase.org/). Many miRNAs are differentially expressed in brains of young nurse bees and old foragers (Behura & Whitfield, 2010; F. Liu et al., 2012), and also among castes (Ashby, Foret, Searle, & Maleszka, 2016). Target

prediction analyses indicated that the differentially expressed miRNAs act in a complex regulatory network and contribute to the differences in honey bee physiology and behavior (Liu et al., 2017; Macedo et al., 2016). Honey bee miR-932 regulates the expression of *Act5C* in the brain, thus modulating long-term memory (Cristino et al., 2014). miR-34-5p may act in early embryogenesis and segmentation by regulating cytoskeleton and pair-rule genes (Freitas, Pires, Claudianos, Cristino, & Simoes, 2017). Higher miR-279a levels in foragers' brain attenuates the sucrose responsiveness, which influences foraging choices of collecting nectar (Liu et al., 2017). Work on bumble bee, *Bombus terrestris* identified 101 miRNA genes, and work in the fig wasp (*Ceratosolen solmsi*) identified several miRNAs in pupae (Y. K. Wang et al., 2017), while 53 miRNA genes were identified in *N. vitripennis* (Werren et al., 2010). We infer that miRNAs serve in a broad range of insect biological processes.

In this study, we sequenced eight small RNA libraries, and identified 75 known and 119 novel miRNA genes in *P. puparum*. We found some developmental and sexua- biased miRNAs, which may act in *P. puparum*. Our results enlarge the number of known miRNA genes in parasitoid wasps, from which we infer these genes are fundamental aspects of their biology.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and small RNA library construction

Laboratory cultures of *P. puparum* were fed on 20% (vol/vov) honey solution, and reared at 25°C under 14L:10D (Cai et al., 2004; Z. Zhang, Ye, Cai, & Hu, 2005). Each female, 2-3 days after eclosion, was allowed to parasitize a *P. rapae* pupa for 12 hr. Whole offspring in embryo (0–12 hr), larval (4 days), white pupal (7 d), and newly emerged adult (14 d) stages from virgins (producing male offspring only, haploid individuals) and mated females (producing mixed-sex offspring, haploid and diploid individuals mixed) were separately collected and homogenized in TRIzol® Reagent (Invitrogen). Total RNA was extracted according to the manufacture's protocol. To generate a sRNA library, 3 μg of total RNA was used for adaptor ligation in both ends using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB), followed by reverse transcription using M-MuLV Reverse Transcriptase (RNase H^-). The polymerase chain reaction (PCR) amplification was performed using LongAmp Taq 2x Master Mix. The PCR products ranging in size from 140 to 160 bp were recovered and sequenced using the Illumina Hi-seq 2500 system.

2.2 | Bioinformatic analyses

The raw reads were processed by FASTX-Toolkit to discard low quality reads, trim the adapter sequence, and collapse identical sequences into unique sequence. The unique reads with low copy number (fewer than 10) were discarded and remaining reads were aligned to the assembly of *P. puparum* genome sequences using bowtie software with no mismatch allowed. Filtered reads were compared to Rfam and RepBase databases to remove noncoding RNAs (rRNA, tRNA, snRNA, snoRNA) and repetitive RNAs using BLASTN ($e < 0.01$). The reads in length 18-25 nt were mapped to all insect reported miRNAs in miRBase 21 using Bowtie (Langmead, Trapnell, Pop, & Salzberg, 2009) with one mismatch allowed. All the mapped reads were clustered based on sequence similarity using CD-HIT (Li & Godzik, 2006) with 100% identity. The predominant reads in each cluster were taken as the known miRNAs and were classified into miRNA families based on the reference miRNAs in miRBase. The known miRNA, together with the remaining reads were then processed by miRDeep2 (Friedlander, Mackowiak, Li, Chen, & Rajewsky, 2012) and Mireap (http://sourceforge.net/projects/mireap/) to predict novel miRNAs and precursors of both known and novel miRNAs. The criteria for Mireap were at least 18 matched base pairs with maximal asymmetry of duplex set to 4. For miRDeep2, default parameters were used.

2.3 | Comparison of miRNA expressions between libraries of different sexes and developmental stages

MiRNA reads in each library were counted by miRDeep2 and the expression levels were normalized to reads per million (RPM). miRNAs differentially expressed between libraries were identified using DEGseq (L. Wang, Feng, Wang, Wang, & Zhang, 2010) package from Bioconductor release 3.3. Q value < 0.001, $\log_2$(fold change) > 1 and RPM > 1,000 in at least one library were set as the threshold for significant differential expression.

2.4 | Validation of miRNAs by reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Reverse transcription reactions were conducted using miScript II RT Kit (Qiagen, Germany) according to the manufacture's protocol. Each reaction (20 μl) contains 1 μg total RNA, 4 μl 5× miScript HiSpec Buffer, 2 μl 10× Nucleics Mix, 2 μl miScript Reverse Transcriptase Mix, and RNase-free water. The reactions were incubated at 37°C for 60 min, 95°C for 5 min, and then held at 4°C. The PCR was conducted using Takara LA Taq, each reaction (50 μl) contains 0.5 μl Takara LA Taq (5 U/μl), 5 μl 10× LA PCR buffer, 8 μl dNTP Mixture, 10 ng cDNA, 2 μl specifically designed primer (10 μM; Table S1), 2 μl 10× miScript Universal Primer (Qiagen), and double distilled H_2O. The PCR products were cloned into pGEM-T Easy vectors (Promega, China) and sequenced. The qRT-PCR was performed using the Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad) with miScript SYBR® Green PCR Kit (Qiagen). The reaction (25 μl) for quantitative PCR (qPCR) contained 10 ng cDNA, 12.5 μl 2× QuantiTect SYBR Green PCR Master Mix, 2.5 μl 10× miScript Universal Primer, 1.5 μl specifically designed primer (10 μM; Table S1), and RNase-free water. All reactions were performed under the following conditions: 95°C for 15 min, 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. To verify the specificity of the amplification for each primer pair, a dissociation curve was included from 60°C to 95°C at the end of each qPCR run. The quantitative variation for each gene was calculated using a relative quantitative method ($-2^{\Delta\Delta C_t}$) for three independent biological samples (Livak & Schmittgen, 2001).

2.5 | Target prediction and enrichment analysis

Reads from *P. puparum* transcriptomic libraries previously constructed by our laboratory (unpublished data) were assembled with Cufflinks (Trapnell et al., 2012) based on the genome sequence. The resulting transcripts were analyzed with Transdecoder (http://transdecoder.github.io) and 3′-UTR sequences were retrieved for miRNA target prediction. We used three target prediction algorithms with default parameters, miRanda (www.microrna.org/; Enright et al., 2003), RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/; Kruger & Rehmsmeier, 2006), and TargetScan (www.targetscan.org/; Lewis, Burge, & Bartel, 2005). Genes jointly predicted by the three algorithms were retained. Gene ontology (GO) enrichment analyses were based on GeneMerge software v1.4, with a filter threshold of corrected p value ≤ 1×10^{-3} and FDR ≤ 0.5% (Castillo-Davis & Hartl, 2003).

2.6 | Statistical analyses

For qPCR results, data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparison test ($p < .05$). The read counts between known and novel miRNAs, 5p and 3p mature miRNAs were analyzed using Student's t test ($p < .05$). All statistical analyses were performed using Data Processing System software (version 14.50; Tang & Zhang, 2013).

3 | RESULTS AND DISCUSSION

3.1 | Overview of small RNA libraries

Eight small RNA libraries produced a combined library with 106,047,094 raw reads in total, among which 12,535,867 reads in male embryos (ME), 14,568,510 in male larvae (ML), 12,517,474 male pupae (MP), and 11,277,840 in male adults (MA). We found 15,464,053 reads in mixed-sex embryos (MSE), 13,403,908 in mixed-sex larvae (MSL), 11,317,031 in mixed-sex pupae (MSP), and 14,962,411 in female adults (FA; Table 1). These data have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession number PRJNA557099. After removing low quality sequences, 90,688,064 clean reads of 18–35 nucleotides (nt) were obtained. The combined library had distribution of small RNAs similar to many insect species, such as *B. mori* (Jagadeeswaran et al., 2010), *Manduca sexta* (X. Zhang et al., 2012) and *Bactrocera dorsalis* (Huang et al., 2014) with a main histogram bar at 22 nt representing miRNAs and smaller bars at 26–28 nt pertaining to PIWI-interacting RNAs (piRNAs; Figure 1). But the length distribution within the eight individual libraries showed that the abundances of small RNAs varied between sexes and among developmental stages (Figure S1). For example, FA histogram bars at 26–28 nt (Panel H) were higher, compared with MA (Panel D). Also, in embryos, the abundance of miRNAs were much lower than piRNAs, which regulate gametogenesis and embryonic development (Ishizu, Siomi, & Siomi, 2012). We divided the remaining reads into categories by blasting to the RepBase and Rfam database. We found low percentages of ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) suggesting that the small RNA libraries were of high quality with little degradation (Table 1).

3.2 | Identification of known and novel miRNAs from *P. puparum*

We identified 75 known miRNA genes coding 125 mature miRNAs (Table S2) in the libraries of ME (54), ML (95), MP (121), and MA (113). We found miRNAs in MSE (71), MSL (107), MSP (122), and FA (117). We did not find five miRNA genes identified in some or all *Nasonia* species: miR-33, miR-219, miR-928, miR-3478, and miR-iab-8, although there were 25 other known miRNAs in *P. puparum*, which were not in *Nasonia* (Table S3). Nearly all miRNAs in *P. puparum* were also present in *A. mellifera* except miR-2779 (Table S3). miR-276-3p is the most abundant, followed by miR-9-5p, and 14 others, with read counts ranging from 170,278 to 4,792,444 (Table S4). We classified miRNAs into families based on the reference miRNAs in miRBase: 65 known miRNAs belonged to 53 families. Using miRDeep2 and Mireap, we predicted the precursors of almost all known miRNAs except miR-278 and miR-2779, probably due to incomplete assembly of the genome. We verified the expression of these two (Figure 2a).

The remaining sequences, which did not map to the insect miRNAs in miRBase were used to identify novel miRNAs. Using miRDeep2 and Mireap, 129 novel mature miRNAs originating from 119 precursors (Table S5) were obtained; the negative folding free energies of their secondary structures range from −59.4 to −20.1 kcal/mol (Data not shown). The presence of four novel miRNAs predicted were verified by RT-PCR (Figure 2a) and their secondary structures are shown in Figure 2b. Among novel miRNAs, mature miRNAs were detected in the library of ME (91), ML (33), MP (47), MA (61), MSE (105), MSL (36), MSP (48), and FA (106). Novel miRNAs populations were present in the two embryo libraries (58% of the miRNAs), 13% in larval libraries, 12% in pupal libraries, and 17% in adult libraries (Figure 3a). Some embryonic progresses such as anterior–posterior and dorso-ventral axis formation differ among species (Fonseca, Lynch, & Roth, 2009; Lynch, Brent, Leaf, Pultz, & Desplan, 2006; Sachs et al., 2015). We infer that the novel miRNAs in embryos may act in embryonic development. The read counts of novel miRNAs (median: 1,100) were significantly lower than known miRNAs (median: 32,892; $p < .0001$, $t = 11.147$, $df = 164$; Figure 3b). We identified 108 miRNA genes (56% of the miRNAs; including 50 known and 58 novel miRNAs) with 5p and 3p mature miRNAs, 40 miRNAs (20%; including 14 known and 26 novel miRNAs) with a 5p, but no 3p arm and 46 miRNAs (24%; including 11 known miRNAs and 35 novel miRNAs) with a 3p, but no 5p arm (Figure 3c). The

TABLE 1 Summaries of eight *Pteromalus puparum* sRNA libraries

	ME	ML	MP	MA	MSE	MSL	MSP	FA	Combined
Raw reads	12,535,867	14,568,510	12,517,474	11,277,840	15,464,053	13,403,908	11,317,031	14,962,411	106,047,094
Clean reads	12,212,667	13,859,068	12,075,789	10,900,798	15,098,424	13,065,008	11,038,307	14,592,800	102,842,861
Reads after length filter	11,743,461	10,493,075	9,614,758	9,279,170	14,435,443	10,680,067	10,404,651	14,037,439	90,688,064
Mapped reads	10,506,276	5,416,155	8,837,862	8,757,246	12632391	7,504,333	10,072,949	13,514,327	77,241,539
rRNA	52,006	204,157	104,728	37,413	75193	284,403	89,796	28,419	876,115
tRNA	0	33	20	0	0	425	0	0	488
snRNA	2,623	2,041	2,816	5,821	4648	1,977	3,707	4,197	27,830
snoRNA	417	9,158	6,109	4,405	1583	13,793	9,586	4,347	49,398
Repeat	649,338	33,153	105,730	140,872	882820	54,216	143,614	627,006	2,636,749
Exon	413,830	423,428	459,920	1,124,202	433204	609,016	564,401	679,837	4,707,838
Intron	2,613,069	867,153	603,048	1,142,984	3009945	1,148,624	925,880	2,521,631	12,832,334

Abbreviations: FA, female adults; MA, male adults; ME, male embryos; ML, male larvae; MP, male pupae; MSE, mixed-sex embryos; SL, mixed-sex larvae; MSP, mixed-sex pupae; rRNA, ribosomal RNA; sRNA, small RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; tRNA, transfer RNA.

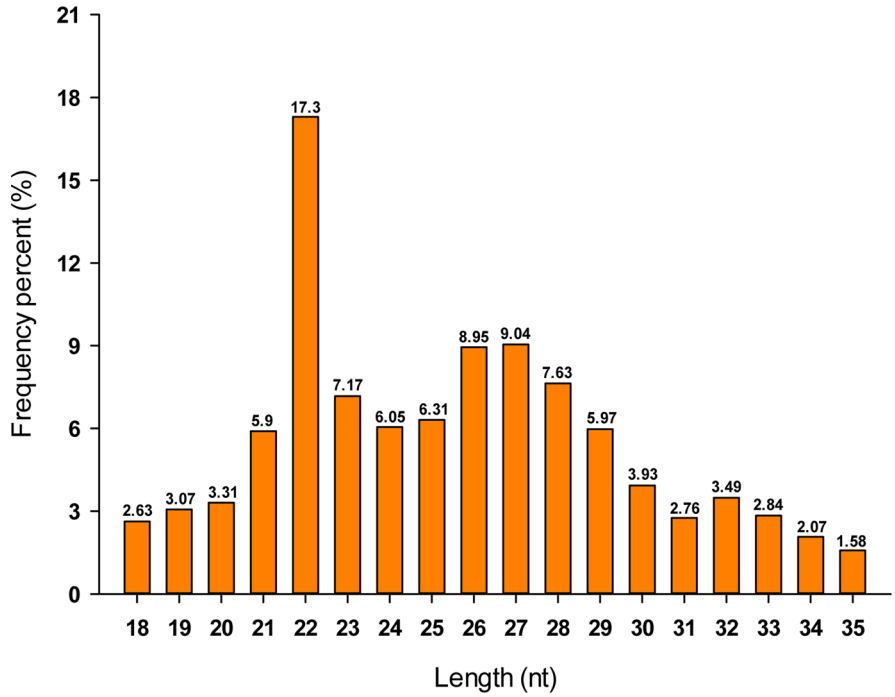

FIGURE 1 Length distribution of small RNAs in eight sRNA libraries combined. The histogram bars represent the frequencies, as percentages, of RNAs of lengths (nt) indicated on the *X* axis. The numbers on top of the bars also show the frequency percentages indicated on the *Y* axis

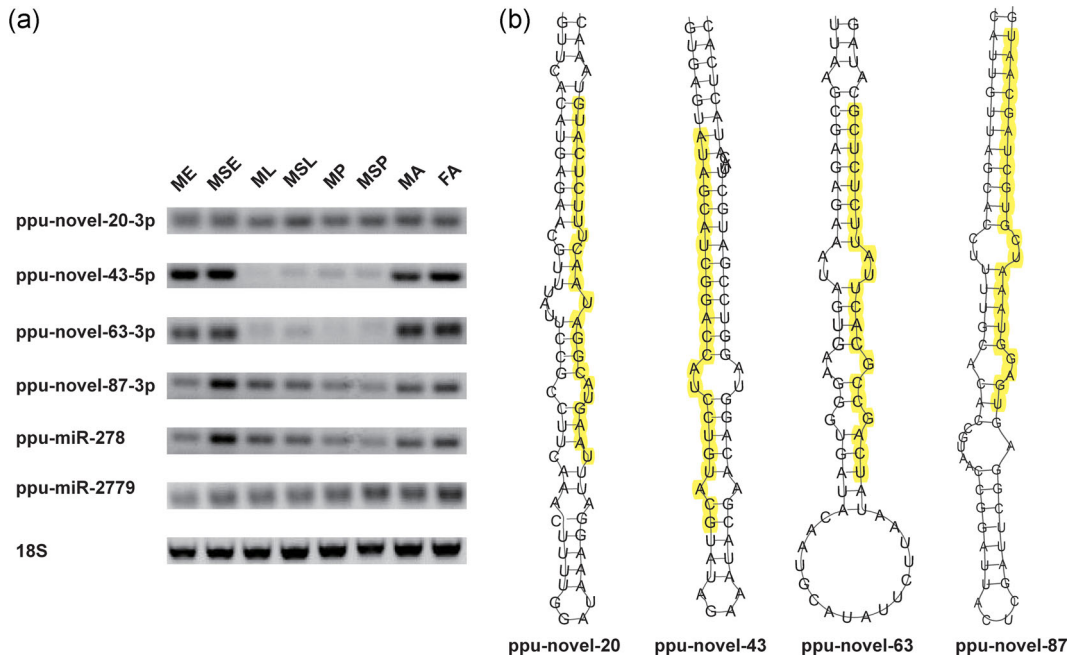

FIGURE 2 Prediction and validation of miRNAs in *Pteromalus puparum*. (a) Validation of novel and two known miRNAs by RT-PCR. The *18S* was used as the reference gene. (b) Calculated folding structure of four predicted novel miRNAs precursor in *P. puparum*. The sequences highlighted in yellow represent the major arms of the miRNA genes. FA, female adults; MA, male adults; ME, male embryos; miRNA, microRNA; ML, male larvae; MP, male pupae; MSE, mixed-sex embryos; MSL, mixed-sex larvae; MSP, mixed-sex pupae; RT-PCR, reverse transcription polymerase chain reaction

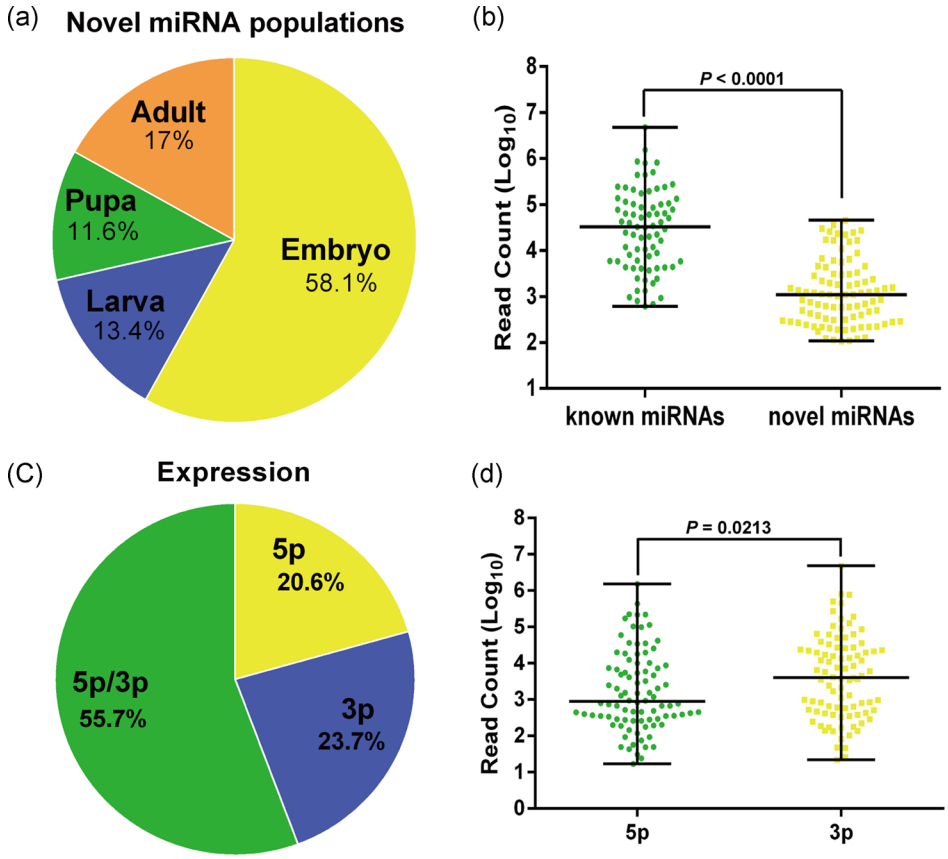

FIGURE 3 Characterization of *Pteromalus puparum* microRNA (miRNA). (a) Distribution of novel miRNA populations through development. (b) Read counts of known (green) and novel (yellow) miRNAs. The horizontal lines depict the median with range. (c) Mature miRNA on duplex. (d) Read counts of miRNAs with detectable 5p (green) and 3p (yellow) mature miRNAs. The horizontal lines depict the median with range

abundances of 3p miRNAs (median: 4,040.5) in *P. puparum* are significantly higher than 5p miRNAs (median, 888; $p = .0213$, $t = 2.323$, $df = 178$; Figure 3d), this is consistent with miRNA expression profiles observed in most of the species (Etebari & Asgari, 2016).

3.3 | Genomic clusters of miRNAs

miRNAs lying within a distance of 10 kb of each other are defined as miRNA clusters (Marco, Ninova, Ronshaugen, & Griffiths-Jones, 2013). In *P. puparum*, 26 miRNA clusters including 86 miRNAs (44%) resides in 18 scaffolds, among which Scaffold_0, Scaffold_25, and Scaffold_553 exist in at least two clusters (Table S6). Among the clusters, all 11 clusters made up of known miRNAs can also be found in *A. mellifera*, and 14 clusters are made up of only novel miRNAs identified in this study. The miRNA cluster-19 has the largest number of miRNA precursors among the clusters, it contains 14 miRNA precursors residing in a 3.3 kb region of Scaffold_260 and codes for six novel miRNAs. Cluster 14 is the second largest cluster with six miRNAs, which occurs in several insect species, but not *B. mori* and *Drosophila* species (Chang et al., 2016). In this study, we found that miRNAs in more than half of the clusters exhibit a consistent expression pattern (Figure 4), predominantly expressed in a specific developmental stage. The well-studied miRNA Cluster-21 (let-7-complex), implicated in metamorphosis was mainly expressed in pupae.

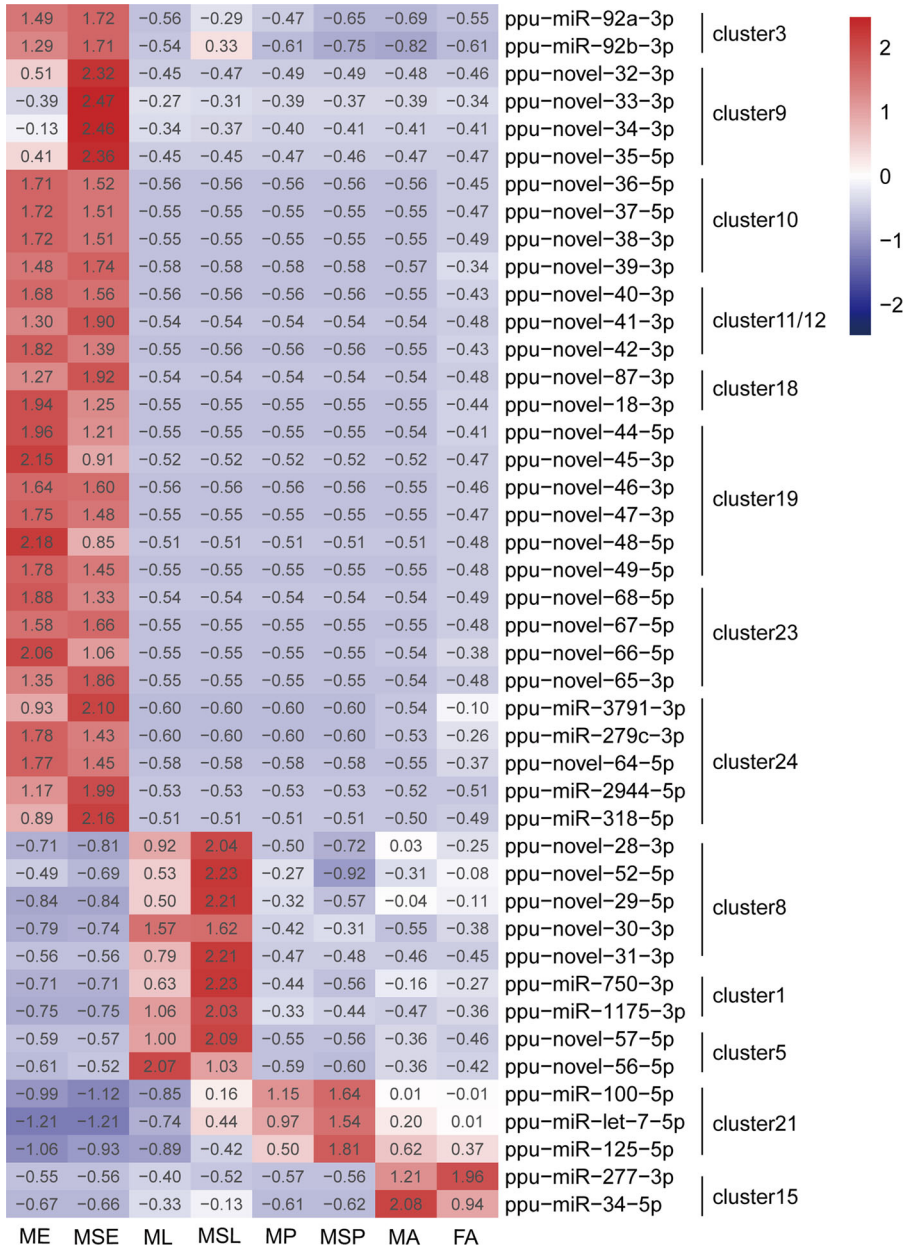

FIGURE 4 Expression profiles of miRNA clusters across the indicated libraries. The numbers indicate $\log_2$RPM values for the miRNAs. The colors in the heat map indicate relative expression: red representing higher expression values and blue representing lower values. FA, female adults; MA, male adults; ME, male embryos; miRNA, microRNA; ML, male larvae; MP, male pupae; MSE, mixed sex embryos; MSL, mixed-sex larvae; MSP, mixed sex pupae

3.4 | Developmental and sex-biased expression of miRNAs

Comparing the ME and ML libraries and the MSE and MSL libraries simultaneously, we showed 48 miRNAs, including 41 novel miRNAs, were consistently expressed in embryos and 36 miRNAs, including 31 known miRNAs, were consistently expressed in larvae (Table S7). There are substantial differences in miRNA expression patterns

possibly because the novel miRNAs are mainly expressed in embryos. Comparing larval and pupal libraries, we identified 14 miRNAs that preferentially expressed in larvae and six miRNAs expressed in pupae. Besides, 10 miRNAs were biasedly expressed in pupae and 16 miRNAs in adults.

We recorded some sex-biased miRNA expression in all four major life stages (Table S8): 10 miRNAs in embryos, 12 in larvae, eight in pupae, and 14 in adults. Part of these biases have been recorded in other insect species, including *C. solms* pupae (Y. K. Wang et al., 2017), *D. melanogaster* (Marco, 2014), and *B. dorsalis* (Peng, Tariq, Xie, & Zhang, 2016). Besides, we also found that the sex-bias of some miRNAs might be changed during *P. puparum* development. miR-315-5p, for example, was more highly expressed in male larvae than female larvae, but lower in male pupae and adults (Table S8). The qRT-PCR experiments were performed for some miRNAs and we confirmed these patterns seen in sequencing data by qPCR (Figure 5).

3.5 | Target prediction and enrichment analysis

Clean reads from our transcriptomic databases were assembled with Cufflinks and analyzed with Transdecoder. We retrieved 13,745 3′-UTR sequences for target prediction of the 254 mature miRNAs. A total of 2,950,459 miRNA–mRNA interactions were predicted by three target prediction algorithms. Among these, 900,436 interactions were predicted by miRanda, 2,282,160 interactions by RNAhybrid, and 816,795 interactions by TargetScan (Figure 6a). We predicted 211,571 interactions by all three algorithms for analysis, recording a mean of

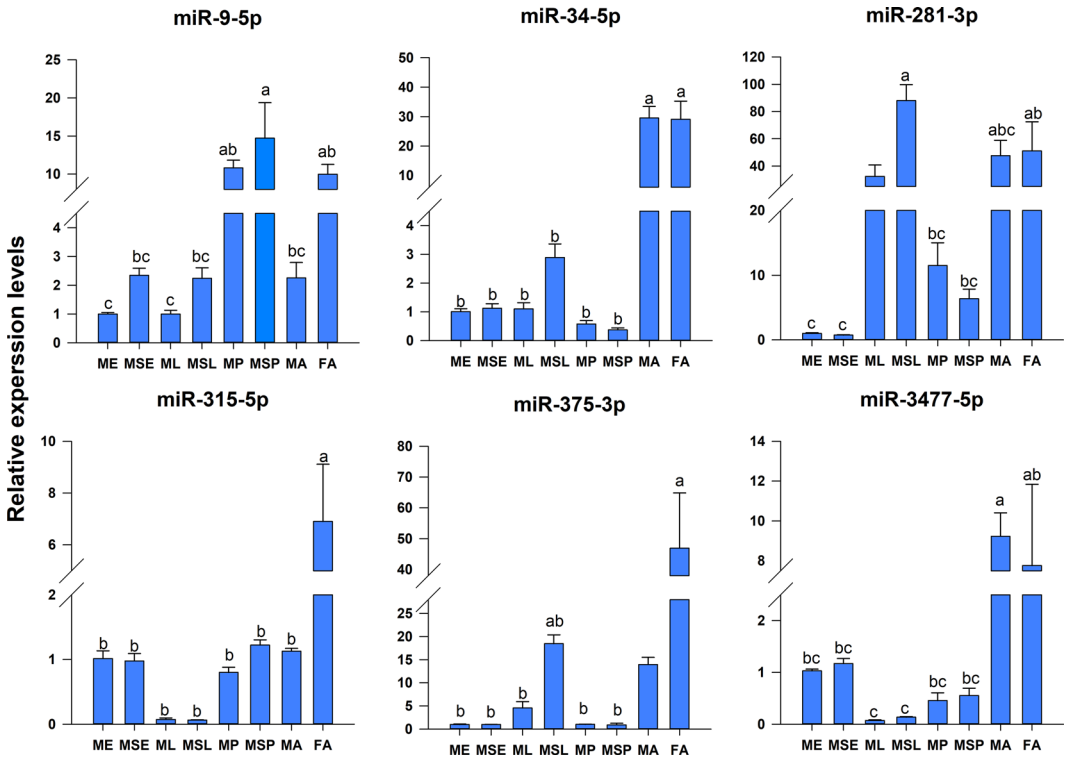

FIGURE 5 Validation of six randomly selected miRNAs. Three biologically independent replicates and three technical replicates were performed. The histogram bars indicate mean relative accumulation of mRNA and the error bars indicate SEM. FA, female adults; MA, male adults; ME, male embryos; miRNA, microRNA; ML, male larvae; MP, male pupae; mRNA, messenger RNA; MSE, mixed-sex embryos; MSL, mixed-sex larvae; MSP, mixed-sex pupae; SEM, standard error of the mean

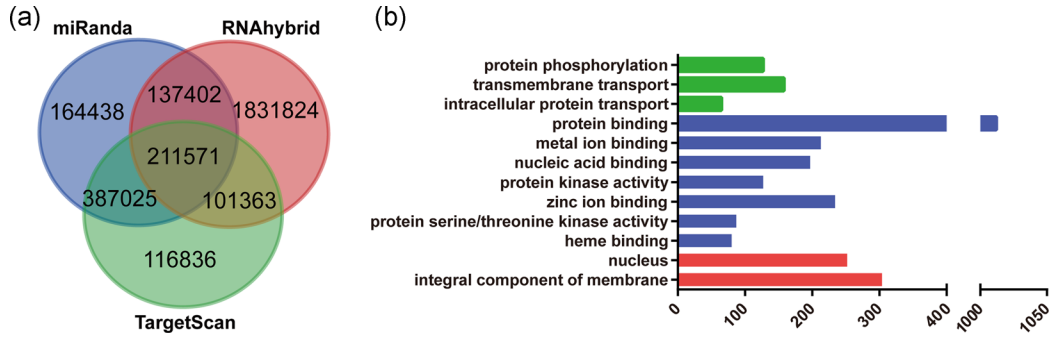

FIGURE 6 Target prediction and GO enrichment. (a) miRNA–mRNA interactions predicted by the three indicated algorithms. The numerals show the number of gene targets identified by each algorithm. (b) The histogram bars represent the gene numbers of enriched GO terms in molecular function (green), biological process (blue), and cellular component (red) categories. GO, Gene Ontology; miRNA, microRNA; mRNA, messenger RNA

833 target genes for each miRNA. About 60% or 8,534 transcripts were predicted to have at least one miRNA-binding site. Gene Ontology (GO) term analysis of target genes showed that protein phosphorylation (GO:0006468), transmembrane transport (GO:0055085), and intracellular protein transport (GO:0006886) were the main terms for biological process (Figure 6b). Protein binding (GO:0005515) and integral component of the membrane (GO:0016021) had the most genes in another two categories, separately.

ACKNOWLEDGMENTS

We appreciate the comments on this manuscript from professor John H. Werren at University of Rochester. This study was supported by the Major International (Regional) Joint Research Project of NSFC (31620103915), the Key Program of National Natural Science Foundation of China (31830074), the National Key R&D Program of China (No. 2017YFD0200400), the Program for Chinese Outstanding Talents in Agricultural Scientific Research of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, the Program for Chinese Innovation Team in Key Areas of Science and Technology of the Ministry of Science and Technology of the People's Republic of China (2016RA4008), and the Natural Science Foundation of Zhejiang Province of China (LY18C140001). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. All programs and services of the US Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

ORCID

Gongyin Ye http://orcid.org/0000-0002-5983-3061
Qi Fang http://orcid.org/0000-0003-4150-3408

REFERENCES

Ashby, R., Forêt, S., Searle, I., & Maleszka, R. (2016). MicroRNAs in honey bee caste determination. *Scientific Reports*, *6*, 18794. https://doi.org/10.1038/srep18794

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281–297.

Behura, S. K., & Whitfield, C. W. (2010). Correlated expression patterns of microRNA genes with age-dependent behavioural changes in honeybee. *Insect Molecular Biology*, *19*(4), 431–439. https://doi.org/10.1111/j.1365-2583.2010.01010.x

Belles, X. (2017). MicroRNAs and the evolution of insect metamorphosis. *Annual Review of Entomology*, *62*, 111–125. https://doi.org/10.1146/annurev-ento-031616-034925

Cai, J., Ye, G., & Hu, C. (2004). Parasitism of *Pieris rapae* (Lepidoptera: Pieridae) by a pupal endoparasitoid, *Pteromalus puparum* (Hymenoptera: Pteromalidae): Effects of parasitization and venom on host hemocytes. *Journal of Insect Physiology*, *50*(4), 315–322. https://doi.org/10.1016/j.jinsphys.2004.01.007

Castillo-Davis, C. I., & Hartl, D. L. (2003). GeneMerge—Post-genomic analysis, data mining, and hypothesis testing. *Bioinformatics*, *19*(7), 891–892.

Caygill, E. E., & Johnston, L. A. (2008). Temporal regulation of metamorphic processes in *Drosophila* by the *let-7* and *miR-125* heterochronic microRNAs. *Current Biology*, *18*(13), 943–950. https://doi.org/10.1016/j.cub.2008.06.020

Chang, Z. X., Tang, N., Wang, L., Zhang, L. Q., Akinyemi, I. A., & Wu, Q. F. (2016). Identification and characterization of microRNAs in the white-backed planthopper, *Sogatella furcifera*. *Insect Science*, *23*(3), 452–468. https://doi.org/10.1111/1744-7917.12343

Chen, W., Liu, Z., Li, T., Zhang, R., Xue, Y., Zhong, Y., … Zhao, Z. (2014). Regulation of *Drosophila* circadian rhythms by miRNA let-7 is mediated by a regulatory cycle. *Nature Communications*, *5*, 5549. https://doi.org/10.1038/ncomms6549

Cristino, A. S., Barchuk, A. R., Freitas, F. C. P., Narayanan, R. K., Biergans, S. D., Zhao, Z., … Claudianos, C. (2014). Neuroligin-associated microRNA-932 targets actin and regulates memory in the honeybee. *Nature Communications*, *5*, 5529. https://doi.org/10.1038/ncomms6529

Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., & Marks, D. S. (2003). MicroRNA targets in *Drosophila*. *Genome Biology*, *5*(1), R1. https://doi.org/10.1186/gb-2003-5-1-r1

Etebari, K., & Asgari, S. (2013). Conserved microRNA miR-8 blocks activation of the Toll pathway by upregulating Serpin 27 transcripts. *RNA Biology*, *10*(8), 1356–1364. https://doi.org/10.4161/rna.25481

Etebari, K., & Asgari, S. (2016). Revised annotation of *Plutella xylostella* microRNAs and their genome-wide target identification. *Insect Molecular Biology*, *25*(6), 788–799. https://doi.org/10.1111/imb.12263

Fonseca, R. N., Lynch, J. A., & Roth, S. (2009). Evolution of axis formation: mRNA localization, regulatory circuits and posterior specification in non-model arthropods. *Current Opinion In Genetics & Development*, *19*(4), 404–411. https://doi.org/10.1016/j.gde.2009.04.009

Freitas, F. C. P., Pires, C. V., Claudianos, C., Cristino, A. S., & Simões, Z. L. P. (2017). MicroRNA-34 directly targets pair-rule genes and cytoskeleton component in the honey bee. *Scientific Reports*, *7*, 40884. https://doi.org/10.1038/srep40884

Friedländer, M. R., Mackowiak, S. D., Li, N., Chen, W., & Rajewsky, N. (2012). miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Research*, *40*(1), 37–52. https://doi.org/10.1093/nar/gkr688

Huang, Y., Dou, W., Liu, B., Wei, D., Liao, C. Y., Smagghe, G., & Wang, J. J. (2014). Deep sequencing of small RNA libraries reveals dynamic expression patterns of microRNAs in multiple developmental stages of *Bactrocera dorsalis*. *Insect Molecular Biology*, *23*(5), 656–667. https://doi.org/10.1111/imb.12111

Iliopoulos, D., Hirsch, H. A., & Struhl, K. (2009). An epigenetic switch involving NF-κB, Lin28, Let-7 microRNA, and IL6 links inflammation to cell transformation. *Cell*, *139*(4), 693–706. https://doi.org/10.1016/j.cell.2009.10.014

Ishizu, H., Siomi, H., & Siomi, M. C. (2012). Biology of PIWI-interacting RNAs: New insights into biogenesis and function inside and outside of germlines. *Genes & Development*, *26*(21), 2361–2373. https://doi.org/10.1101/gad.203786.112

Jagadeeswaran, G., Zheng, Y., Sumathipala, N., Jiang, H., Arrese, E. L., Soulages, J. L., … Sunkar, R. (2010). Deep sequencing of small RNA libraries reveals dynamic regulation of conserved and novel microRNAs and microRNA-stars during silkworm development. *BMC Genomics*, *11*, 52. https://doi.org/10.1186/1471-2164-11-52

Kruger, J., & Rehmsmeier, M. (2006). RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Research*, *34*(Web Server issue), W451–W454. https://doi.org/10.1093/nar/gkl243

Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, *10*(3), R25. https://doi.org/10.1186/gb-2009-10-3-r25

Lee, G. J., & Hyun, S. (2014). Multiple targets of the microRNA miR-8 contribute to immune homeostasis in *Drosophila*. *Developmental & Comparative Immunology*, *45*(2), 245–251. https://doi.org/10.1016/j.dci.2014.03.015

Lewis, B. P., Burge, C. B., & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, *120*(1), 15–20. https://doi.org/10.1016/j.cell.2004.12.035

Li, W., & Godzik, A. (2006). Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, *22*(13), 1658–1659. https://doi.org/10.1093/bioinformatics/btl158

Ling, L., Ge, X., Li, Z., Zeng, B., Xu, J., Aslam, A. F. M., … Tan, A. (2014). MicroRNA *Let-7* regulates molting and metamorphosis in the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology*, *53*, 13–21. https://doi.org/10.1016/j.ibmb.2014.06.011

Ling, L., Kokoza, V. A., Zhang, C., Aksoy, E., & Raikhel, A. S. (2017). microRNA-277 targets *insulin-like peptides 7* and *8* to control lipid metabolism and reproduction in *Aedes aegypti* mosquitoes. *Proceedings of the National Academy of Sciences*, *114*(38), E8017–E8024. https://doi.org/10.1073/pnas.1710970114

Liu, F., Peng, W., Li, Z., Li, W., Li, L., Pan, J., … Su, S. (2012). Next-generation small RNA sequencing for microRNAs profiling in *Apis mellifera*: Comparison between nurses and foragers. *Insect Molecular Biology*, *21*(3), 297–303. https://doi.org/10.1111/j.1365-2583.2012.01135.x

Liu, F., Shi, T., Yin, W., Su, X., Qi, L., Huang, Z. Y., … Yu, L. (2017). The *microRNA ame-miR-279a* regulates sucrose responsiveness of forager honey bees (*Apis mellifera*). *Insect Biochemistry and Molecular Biology*, *90*, 34–42. https://doi.org/10.1016/j.ibmb.2017.09.008

Liu, N., Landreh, M., Cao, K., Abe, M., Hendriks, G. J., Kennerdell, J. R., … Bonini, N. M. (2012). The microRNA *miR-34* modulates ageing and neurodegeneration in *Drosophila*. *Nature*, *482*(7386), 519–523. https://doi.org/10.1038/nature10810

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Lozano, J., Montañez, R., & Belles, X. (2015). miR-2 family regulates insect metamorphosis by controlling the juvenile hormone signaling pathway. *Proceedings of the National Academy of Sciences*, *112*(12), 201418522. https://doi.org/10.1073/pnas.1418522112

Lynch, J. A., Brent, A. E., Leaf, D. S., Anne Pultz, M., & Desplan, C. (2006). Localized maternal orthodenticle patterns anterior and posterior in the long germ wasp *Nasonia*. *Nature*, *439*(7077), 728–732. https://doi.org/10.1038/nature04445

Macedo, L. M., Nunes, F. M., Freitas, F. C., Pires, C. V., Tanaka, E. D., Martins, J. R., & Simoes, Z. L. (2016).). MicroRNA signatures characterizing caste-independent ovarian activity in queen and worker honeybees (*Apis mellifera* L.). *Insect Molecular Biology*, *25*(3), 216–226. https://doi.org/10.1111/imb.12214

Marco, A. (2014). Sex-biased expression of microRNAs in *Drosophila melanogaster*. *Open Biology*, *4*, 140024. https://doi.org/10.1098/rsob.140024

Marco, A., Ninova, M., Ronshaugen, M., & Griffiths-Jones, S. (2013). Clusters of microRNAs emerge by new hairpins in existing transcripts. *Nucleic Acids Research*, *41*(16), 7745–7752. https://doi.org/10.1093/nar/gkt534

Mendell, J. T. (2008). miRiad roles for the miR-17-92 cluster in development and disease. *Cell*, *133*(2), 217–222. https://doi.org/10.1016/j.cell.2008.04.001

O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., & Baltimore, D. (2010). Physiological and pathological roles for microRNAs in the immune system. *Nature Reviews Immunology*, *10*(2), 111–122. https://doi.org/10.1038/nri2708

Peng, W., Tariq, K., Xie, J., & Zhang, H. (2016). Identification and characterization of sex-biased microRNAs in *Bactrocera dorsalis* (Hendel). *PLOS One*, *11*(7):e0159591. https://doi.org/10.1371/journal.pone.0159591

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., & Ruvkun, G. (2000). The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, *403*(6772), 901–906. https://doi.org/10.1038/35002607

Roncarati, R., Viviani Anselmi, C., Losi, M. A., Papa, L., Cavarretta, E., Da Costa Martins, P., & Condorelli, G. (2014). Circulating miR-29a, among other up-regulated microRNAs, is the only biomarker for both hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy. *Journal of the American College of Cardiology*, *63*(9), 920–927. https://doi.org/10.1016/j.jacc.2013.09.041

Rubio, M., & Belles, X. (2013). Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolan metamorphosis. *Journal of Insect Physiology*, *59*(11), 1089–1094. https://doi.org/10.1016/j.jinsphys.2013.09.003

Rupaimoole, R., Calin, G. A., Lopez-Berestein, G., & Sood, A. K. (2016). miRNA deregulation in cancer cells and the tumor microenvironment. *Cancer Discovery*, *6*(3), 235–246. https://doi.org/10.1158/2159-8290.cd-15-0893

Sachs, L., Chen, Y. T., Drechsler, A., Lynch, J. A., Panfilio, K. A., Lassig, M., & Roth, S. (2015). Dynamic BMP signaling polarized by Toll patterns the dorsoventral axis in a hemimetabolous insect. *eLife*, *4*, e05502. https://doi.org/10.7554/eLife.05502

Tang, Q. Y., & Zhang, C. X. (2013). Data processing system (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. *Insect Science*, *20*(2), 254–260. https://doi.org/10.1111/j.1744-7917.2012.01519.x

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., & Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, *7*(3), 562–578. https://doi.org/10.1038/nprot.2012.016

Varghese, J., Lim, S. F., & Cohen, S. M. (2010). *Drosophila* miR-14 regulates insulin production and metabolism through its target, *sugarbabe*. *Genes & Development*, *24*(24), 2748–2753. https://doi.org/10.1101/gad.1995910

Wang, L., Feng, Z., Wang, X., Wang, X., & Zhang, X. (2010). DEGseq: An R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*, *26*(1), 136–138. https://doi.org/10.1093/bioinformatics/btp612

Wang, Y. K., Li, J. P., Li, T. T., Liu, R., Jia, L. Y., Wei, X. Q., & Huang, D. W. (2017). Comparative profiling of microRNAs and their association with sexual dimorphism in the fig wasp *Ceratosolen solmsi*. *Gene*, *633*, 54–60. https://doi.org/10.1016/j.gene.2017.08.030

Werren, J. H., & Loehlin, D. W. (2009). The parasitoid wasp *Nasonia*: An emerging model system with haploid male genetics. *Cold Spring Harbor Protocols*, *2009*(10), https://doi.org/10.1101/pdb.emo134. pdb.emo134.

Werren, J. H., Richards, S., Desjardins, C. A., Niehuis, O., Gadau, J., Colbourne, J. K., & Gibbs, R. A. (2010). Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science*, *327*(5963), 343–348. https://doi.org/10.1126/science.1178028

Xiong, X. P., Kurthkoti, K., Chang, K. Y., Li, J. L., Ren, X., Ni, J. Q., & Zhou, R. (2016). *miR-34* modulates innate immunity and ecdysone signaling in *Drosophila*. *PLOS Pathogens*, *12*(11):e1006034. https://doi.org/10.1371/journal.ppat.1006034

Yan, Z., Fang, Q., Wang, L., Liu, J., Zhu, Y., Wang, F., & Ye, G. (2016). Insights into the venom composition and evolution of an endoparasitoid wasp by combining proteomic and transcriptomic analyses. *Scientific Reports*, *6*, 19604. https://doi.org/10.1038/srep19604

Zhang, X., Zheng, Y., Jagadeeswaran, G., Ren, R., Sunkar, R., & Jiang, H. (2012). Identification and developmental profiling of conserved and novel microRNAs in *Manduca sexta*. *Insect Biochemistry and Molecular Biology*, *42*(6), 381–395. https://doi.org/10.1016/j.ibmb.2012.01.006

Zhang, Y., Zhao, B., Roy, S., Saha, T. T., Kokoza, V. A., Li, M., & Raikhel, A. S. (2016). microRNA-309 targets the Homeobox gene *SIX4* and controls ovarian development in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(33), E4828–E4836. https://doi.org/10.1073/pnas.1609792113

Zhang, Z., Ye, G. Y., Cai, J., & Hu, C. (2005). Comparative venom toxicity between *Pteromalus puparum* and *Nasonia vitripennis* (Hymenoptera: Pteromalidae) toward the hemocytes of their natural hosts, non-target insects and cultured insect cells. *Toxicon*, *46*(3), 337–349. https://doi.org/10.1016/j.toxicon.2005.05.005

Zhu, J. Y., Ye, G. Y., Dong, S. Z., Fang, Q., & Hu, C. (2009). Venom of *Pteromalus puparum* (Hymenoptera: Pteromalidae) induced endocrine changes in the hemolymph of its host, *Pieris rapae* (Lepidoptera: Pieridae). *Archives of Insect Biochemistry and Physiology*, *71*(1), 45–53. https://doi.org/10.1002/arch.20304

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Xiao S, Wang B, Li K, et al. Identification and characterization of miRNAs in an endoparasitoid wasp, *Pteromalus puparum*. *Arch Insect Biochem Physiol*. 2019;e21633. https://doi.org/10.1002/arch.21633