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Identification of differentially expressed microRNAs in *Culex pipiens* and their potential roles in pyrethroid resistance

Shanchao Hong¹, Qin Guo¹, Weijie Wang¹, Shengli Hu¹, Fujin Fang¹, Yuan Lv¹, Jing Yu¹, Feifei Zou¹, Zhentao Lei¹, Kai Ma¹, Lei Ma¹, Dan Zhou¹, Yan Sun¹, Donghui Zhang¹, Bo Shen¹, and Changliang Zhu^{1,*}

¹Department of Pathogen Biology, Nanjing Medical University, Nanjing, Jiangsu, 210029, P. R. China

Abstract

Pyrethroids are the major class of insecticides used for mosquito control. Excessive and improper use of insecticides, however, has resulted in pyrethroid resistance, which has become a major obstacle for mosquito control. The development of pyrethroid resistance is a complex process involving many genes, and information on post-transcription regulation of pyrethroid resistance is lacking. In this study, we extracted RNA from mosquitoes in various life stages (fourth-instar larvae, pupae, male and female adult mosquitoes) from deltamethrin-sensitive (DS) and resistant (DR) strains. Using Illumina sequencing, we obtained 13760296 and 12355472 reads for DS-strains and DR-strains, respectively. We identified 100 conserved miRNAs and 42 novel miRNAs derived from 21 miRNA precursors in *Culex pipiens*. After normalization, we identified 28 differentially expressed miRNAs between the two strains. Additionally, we found that cpp-miR-71 was significant down regulated in female adults from the DR-strain. Based on microinjection and CDC Bottle Bioassay data, we found that cpp-miR-71 may play a contributing role in deltamethrin resistance. The present study provides the firstly large-scale characterization of miRNAs in *Culex pipiens* and provides evidence of post-transcription regulation. The differentially expressed miRNAs between the two strains are expected to contribute to the development of pyrethroid resistance.

Keywords

Culex pipiens; pyrethroid resistance; expression profile; differentially expressed microRNA; microinjection; American CDC Bottle Bioassay

1. Introduction

Mosquito-borne diseases, such as malaria, dengue fever, yellow fever, filariasis and encephalitis, dramatically jeopardize public health and impede economic development

*To whom correspondence should be addressed: Tel: +862586862793; Fax: +862586862793; clzhu@njmu.edu.cn (CZ).

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(Nkya et al., 2013). Between 2006 and 2009, there were 5794 cases of Japanese encephalitis reported in China and India each year, with the majority of cases occurring in children aged 0–14 years (Campbell et al., 2011).

Historically, chemical compounds have been the principal method of mass prevention for mosquito-borne diseases (Sun et al., 2013). Pyrethroid, fourth generation synthetic insecticide, modifies the function of voltage sensitive sodium channels (Soderlund and Knipple, 2003) which disrupts the nervous system (Chen et al., 2010). Deltamethrin, a representative pyrethroid insecticide, is commonly used for the indoor residual spraying and impregnation of bed nets due to its low toxicity (Xu et al., 2014). Excessive and improper application of pyrethroids, however, has caused resistance, and mosquito-borne diseases are now resurgent (Hart et al., 2014).

Insecticide resistance results from polygenic inheritance (David et al., 2005). Mutation of sodium channels, activation of detoxification enzymes and upregulation of other genes could contribute to the development of pyrethroid resistance (Hemingway et al., 2004; Hemingway and Ranson, 2000). Expression of these genes is regulated at both transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) are known to be a key component in post-transcriptional gene expression regulation in many species.

MiRNAs are endogenous noncoding RNAs with lengths of 19–23 nucleotides. Upon Dcr-1 cleavage of pre-miRNA to form a mature miRNA/miRNA* duplex, one strand is loaded into RNA-induced silencing complex (Wostenberg et al., 2012) and the other strand is degraded (Leclercq et al., 2013). MiRNAs regulate gene expression at the post-transcription level via base pairing to target sites within messenger RNAs (mRNAs) (Lucas and Raikhel, 2013). In most cases, the miRNA “seed sequence” (nucleotides 2–8 at the 5′ end of miRNA) binds to the 3′ untranslated regions (3′UTR) of mRNA resulting in regulation of mRNA translation or mRNA degradation (Bartel, 2004). Since miRNAs were first discovered in *Caenorhabditis elegans*, multiple miRNAs have been shown to play considerable roles in regulating cellular differentiation, proliferation, apoptosis and development in vertebrates, invertebrates, plants and viruses (Ameres and Zamore, 2013). In *Anopheles gambiae*, the expression profile of miRNAs was examined and four miRNAs were found to be affected by the presence of *Plasmodium* (Winter et al., 2007). In *An. stephensi*, 27 miRNAs have been identified and distinct patterns of miRNAs expression were revealed from embryo to adult stages (Gregory et al., 2011). Sixty-five miRNAs in the *Aedes albopictus* C7/10 cell line and 77 miRNAs in *Cx. quinquefasciatus* were also detected and miR-92 and miR-989 showed significant changes following WNV infection (Skalsky et al., 2010).

The role of miRNAs in insecticide resistance in mosquito has received little attention. Next-generation sequencing technology makes it possible to precisely identify non-conserved or weakly expressed miRNAs (Yamamoto et al., 2014). The identification and comparison of miRNA expression in deltamethrin-sensitive strains (DS) and deltamethrin-resistant strains (DR) of *Cx. pipiens* would contribute to a better understanding of the mechanism of pyrethroid resistance. In addition, we provide the first evidence that cpp-miR-71 participates in pyrethroid resistance in mosquitoes and confirm interactions between cpp-miR-71 and the 3′UTR of its target gene *cytochrome P450 325BG3 (CYP325BG3)*.

2. Materials and Methods

2.1. Mosquito strains

A DS-strain of *Cx. pipiens* (LC₅₀ was 0.03mg/l) which was never been exposed to any insecticides, was collected from Tang Kou, ShanDong province. Two DR-strains (DR1-strain, DR2-strain) were used in this study. DR1-strain was selected with deltamethrin for more than 10 generations and the resistance index reaches 28.3(LC₅₀ was 0.85mg/l). DR2-strain was subjected to deltamethrin selection for more than 60 generations, the LC₅₀ was up to 7mg/l. All the strains were reared in a humidified insectary at 28–30 °C on a 16 h light/8 h dark cycle.

2.2. *Cx. pipiens* sample preparation for illumina sequencing

Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Dalian, China) from mixed life stages (including fourth-instar larvae, pupae, male and female adults) from DS and DR1-strains. Pupae were collected from varied ages. Male and female adults aged 3–5 days post emergence were collected. Approximately 30 µg of the total RNA was extracted and sent to the Beijing Genome Institute Inc. for sequencing and analysis. About 10 µg total RNA was isolated on a 15% denaturing polyacrylamide gel. Small RNAs ranging from 18 to 30 nt in length were excised and ligated sequentially from 5' to 3' ends. Then adding the adaptor primers to amplify RNAs and about 90 nt fragments were isolated from agarose gels. The extracted DNA was sequenced by Illumina Hi Seq™ 2000. Clean reads were processed after removing contaminated reads and adaptor sequences.

2.3. Small RNA sequence analysis

Small RNA clean reads were mapped to the *Cx. quinquefasciatus* genome with Short Oligonucleotide Alignment Programs (SOAP) (Li et al., 2008), which was closely related mosquito in the same subgenus with *Cx. pipiens*. Only perfect matches were accepted and retained for the next analysis. We aligned all small RNA reads to mature miRNAs and blasted miRNA hairpins in miRBase18.0 and filtered them using an E-value threshold of 0.01. We annotated the other non-coding RNAs to rRNA, scRNA, snoRNA, snRNA and tRNA from Genbank and Rfam (9.1). We also annotated small RNAs according to exon and intron of mRNAs from Vector Base. The remaining unannotated small RNAs were tested by MIREAP (<https://sourceforge.net/projects/mireap/>) for miRNA-coding potential. We took the predominant small RNA from “MIREAP” as the novel representative miRNA. When miRNA or miRNA* had at least 15 reads in the two samples, we classified it into the expressed miRNA.

2.4. Amplification of the miRNA precursors

We extracted genomic DNA from the female adults using the method as previously described (Collins et al., 1987). We designed primers for 36 conserved miRNAs and 12 novel miRNAs according to the sequences of the mature miRNA and miRNA* or the sequences in *Cx. quinquefasciatus* genome using Primer Premier 5.0 (Premier Biosoft International, PaloAlto, CA, USA). The sequences of the primers were in the Table S1. Fragments were amplified by PCR and the products were examined by 2.5% agarose gels.

2.5. Stem-loop quantitative RT-PCR assay

Stem-loop RT-PCR was used to verify the expression levels of miRNAs between the two strains (Tang et al., 2006). Briefly, total RNA from embryos, instar larvae, pupae, male and female adults was extracted by RNAiso Plus reagent, respectively. 2 µg total RNA was reverse-transcribed to cDNA using AMV transcriptase (TaKaRa, Dalian, China) and looped antisense primer. The mixture was incubated at 42°C for 60 minutes and 85 °C for 5 minutes. qRT-PCR was performed on the ABI Prism 7300 HT Sequence Detection system (Applied Biosystem, CA, USA) using FastStart® SYBR Green (Applied Biosystem, CA, USA). Reactions were incubated in a 96-well optical plate at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1min. A melting curve program was run immediately after the PCR and the data was analyzed by 7300 System SDS Software v1.2.1 (Applied Biosystems). Table S2 was the primers for the stem-loop quantitative RT-PCR. The raw threshold cycle (Ct) values were normalized against *U6* standard to obtain normalized Ct values, which were used to calculate relative expression levels in samples using the 2^{-Ct} method (Livak and Schmittgen, 2001).

2.6. Target prediction

It was important to predict miRNA targets in understanding miRNA function. The rules used for target prediction were based on those suggested by Allen et al. (Allen et al., 2005) and Schwab et al. (Schwab et al., 2005). Unfortunately, there was no 3'UTR database available for *Cx. quinquefasciatus*, making it difficult to predict targets for cpp-miRNAs using computational prediction tools. Cytochrome P450 monooxygenase (CYP450) genes have proven particularly important in resistance to insecticides (Schuler and Berenbaum, 2013). Based on transcripts of CYP450 from the *Cx. quinquefasciatus*, the 3'UTR sequence of *CYP325BG3* contained a target site for cpp-miR-71. The partial 3'UTR sequence of the *Cx. pipiens CYP325BG3* gene was obtained by PCR using RACE cDNA. The RACE cDNA was reversed using SMART RACE cDNA Amplification kit (Clontech, Tokyo, Japan). The PCR primer was: forward primer, 5'-ATCGGAATCCATTTTCGTTTT-3', reverse primer, 5'-TTGTAGTCTCCATTTGCCACG-3'.

2.7. Vector construction and luciferase assay

The *CYP325BG3* 3'UTR sequence was amplified from cDNAs from female adults by PCR using the following primers: forward primer, 5'-CCAAGCTTGAAGAGCCTTAAGTCTAATTT-3'; reverse primer, 5'-CGAGTCATCGTCTTATGAAAGACAATA-3'. For its mutagenesis, the sequence complementary to the binding site of cpp-miR-71 seed sequence in its 3'UTR (GTCTTTC) was replaced by CTGTATG. The wild type and muted 3'UTR of *CYP325BG3* were both cloned into PMIR-Report miRNA reporter vector using the *Hind*III and *Sac*I sites. The constructs were validated by sequencing.

The sequence of cpp-miR-71 was: 5'-AGAAAGACATGGGTAGTGAGAT-3'. The miRNA mimic was designed and procured from GenePharma (GenePharma, Shanghai, China) at a concentration of 20 µM. HEK-293 cells were seeded into a 6-well plate for 24h. The cells were divided into four groups, UTR/miR-71mimic, UTR/NC mimic, MUT/miR-71 mimic and MUT/NC mimic. The plasmids were transfected into cells using FuGENE® HD

Transfection Reagent (Promega, WI, USA). The solutions were mixed gently and incubated at room temperature for 15 min. The mixture was layered onto cells gently. 48h after transfection, cells were lysed and subjected to luciferase assay that was performed using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

2.8. Microinjection of female adult mosquito

The cpp-miR-71 mimic was designed and procured from GenePharma (GenePharma, Shanghai, China) at a concentration of 20 μM . The details of the sequences were included in Table S3. Unfed female adults mosquitoes from the DR2-strain at one day post emergence were used for microinjection. The mosquitoes were anaesthetized in the $-20\text{ }^{\circ}\text{C}$ refrigerator for 6 to 8 minutes and injected in the thorax with 0.5 μl (500 nl) of cpp-miR-71 mimic at a final concentration of 20 pmol / μl on ice (Kumar and Puttaraju, 2012; Puthiyakunnon et al., 2013). After microinjection, mosquitoes were placed to small plastic tubes immediately and were allowed to recover with sugar water in a humidified insectary at 28–30 $^{\circ}\text{C}$ on a 16 h light/8 h dark cycle. The negative control (NC) mimic was injected the same volume into mosquitoes and the control group was injected 0.5 μl water pretreated with diethylpyrocarbonate (DEPC-water) at the same time. Three days after recovery, the efficiency of the miRNA mimic microinjection was confirmed using stem-loop quantitative RT-PCR. The sequences of primer of cpp-miR-71 for stem-loop quantitative RT-PCR were in Table S2. The expression levels of target gene *CYP325BG3* were confirmed by qRT-PCR. Its primers were: forward primer, 5'-ATTCTTGCTGAAGGAAGTGGC-3'; reverse primer, 5'-AATCGTGATCGCGTGGTGT-3'.

2.9. American CDC Bottle Bioassay

Unfed female adults of one day post emergence from the DR2-strain were used for microinjection. Then we microinjected miRNA mimic, control mimic and DEPC-water as above. Mosquitoes were put in the recovery tubes after microinjection. Three days later, we examined them using American CDC Bottle Bioassay, a tool for detecting the resistance to insecticides (Aizoun et al., 2013). According to the laboratory guideline of the CDC bottle bioassay, the recommended dosage of deltamethrin did not kill individual mosquito in our first bioassay, we decided to increase the dosage. The diagnostic dose of deltamethrin applied in this study was 4 mg per bottle (250 ml). For each bioassay, 15 to 20 mosquitoes per bottle were exposed to deltamethrin. The control bottle was coated with acetone only and tested with cpp-miR-71 mimic microinjected mosquitoes. The number of dead mosquitoes was recorded every 15 minutes, up to 2 hours. At last the mortality rate was calculated at the diagnostic time.

2.10. siRNA microinjection

Short interfering RNAs (siRNA) targeting *CYP325BG3* were designed and procured from GenePharma (GenePharma, Shanghai, China) at a concentration of 20 μM (The sequences were in Table S4). 0.07 μl of the siRNA and negative control (NC) RNA were microinjected into female adults from the DR2-strain at a final concentration of 5 $\mu\text{g}/\mu\text{l}$ (The procedures of microinjection were described in section 2.8.). The control group was microinjected the same volume of DEPC-water at the same time. Three days after microinjection, the expression levels of target gene *CYP325BG3* were confirmed by qRT-PCR. The relationship

between deltamethrin resistance and *CYP325BG3* was confirmed by American CDC Bottle Bioassay.

3. Results and Discussion

3.1. Small RNA expression profiling in *Cx. pipiens*

In order to examine the role of small RNAs in pyrethroid resistance in *Cx. pipiens*, we collected samples from fourth-instar larvae, pupae, male and female adults from DS and DR1 strains for sequencing. Small RNAs of less than 30 nt were isolated and sequenced. After filtering out sequences shorter than 18 nt and lower quality reads, 13760296 and 12355472 reads of the DS-strain and DR1-strain remained for further analysis. More than 50% of the reads were expected to be insect miRNA. The percentage of miRNA isolated from the DR1-strain was found to be 62.94% while in the DS-strain the percentage of miRNA was 55.35%. The length distribution of these clean reads was summarized to illustrate the compositions of small RNA samples (Fig. S1A, B in Supplementary data). miRNA is normally ~19–23 nt (Bartel, 2004), endogenous siRNA is ~21 nt (Czech et al., 2008), and piRNA is ~27–30 nt (Siomi et al., 2011). MiRNA and endogenous siRNA are similar lengths, however, miRNA has a tendency to begin with uracil (Fig. S1C, D in Supplementary data). We analyzed the first nucleotide bias of small RNAs. The results suggested that majority of the ~19–22 nt small RNAs were miRNA rather than endogenous siRNA. The proportion of small RNAs with a length of ~27–30 nt was much higher in the DS-strain than that in DR1-strain, which suggested that piRNA may also play a role in pyrethroid resistance. Further investigation of these small RNAs could be helpful in improving our understanding of mechanisms of insecticide resistance.

3.2. Conserved and novel miRNAs in *Cx. pipiens*

We aligned small RNA sequences to the miRNA of corresponding species in miRBase (<http://www.mirbase.org/>) and the characteristic hairpin structure of miRNA was predicted by MIREAP. In total, we detected 100 conserved miRNA genes. Among these, 71 genes were present as a single copy in the *Cx. quinquefasciatus* genome, while others had multiple copies distributed on different loci that produced identical mature miRNAs (Table 1). The *Cx. pipiens* miRNA and miRNA* strands mapped with 100% identity to the *Cx. quinquefasciatus*. However, the strand selection differed between the two closely-related mosquito species. In *Cx. quinquefasciatus*, cqu-miR-11, cqu-miR-308, and cqu-miR-998 had more 3p reads than 5p reads. In *Cx. pipiens*, cpp-miR-11 had the similar 5p and 3p reads, while cpp-miR-308, cpp-miR-998 had more 5p reads. One reason for this strand selection difference may be that the miRNAs came from different subgenus. Another potential reason was that samples used for sequencing came from different sources. The samples used for sequencing of *Cx. quinquefasciatus* were from female adults, while the samples from *Cx. pipiens* were obtained from mixed growth stages (fourth-instars larvae, pupae, male and female adults).

In order to identify novel miRNAs, we used MIREAP software to identify potential stem-loop structures in sequences flanking the remaining unannotated reads. The criteria to predict the authenticity of miRNAs was set forth by Berezikov et al. (Berezikov et al., 2011).

This method yielded 42 potential miRNAs derived from 21 miRNA precursors (Table 1). We found the levels of novel miRNAs were far lower than the known conserved miRNAs. This phenomenon was also found in *Honey bees* and *Ae. albopictus* (Guo et al., 2013; Kim et al., 2013). The low expression levels suggested that the novel miRNAs were expressed in specific developmental stages and organs.

The precursors of conserved and novel miRNAs were all predicted from the *Cx. quinquefasciatus* genome. We confirmed these precursors using PCR. We designed 48 pairs of primers for the precursors, 36 pairs for conserved miRNAs and 12 for novel miRNAs. As expected, we amplified products shorter than 100 bp from the *Cx. pipiens* genome (Fig. S2, in Supplementary data). The results showed that the precursors of miRNAs existed in the *Cx. pipiens* genome.

3.3. Differentially expressed miRNAs and determination of candidate miRNA

In total, 13760296 and 12355472 miRNAs reads were obtained in the DS-strain and DR1-strain. We first normalized the expression of miRNAs in two samples and calculated the fold-change and P-value based on the normalized expression. Next, we generated the log₂ ratio figure and scatter plot (Fig. 1). The expression levels of 26 conserved miRNAs and 2 novel miRNAs differed between the two strains. Nineteen miRNAs showed higher expression levels in the DS-strain and nine miRNAs showed higher expression level in the DR1-strain (Table 2).

Stem-loop RT-PCR was used to verify the expression levels of candidate differentially expressed miRNAs (Fig. 2). The results suggested that cpp-miR-2-3p, cpp-miR-13, cpp-miR-285-3p, cpp-miR-4448 and cpp-miR-71 were up-regulated in the DS-strain at most stages. Cpp-miR-965-3p, cpp-miR-317 and cpp-miR-2840 were up-regulated in the DR1-strain at most stages. Cpp-miR-92a, cpp-miR-3781, cpp-miR-184-3p and cpp-miR-1-3p had different expression levels at different life stages.

The differentially expressed miRNAs in the two strains provide a basis for investigation of pyrethroid resistance. MiR-1-3p was specifically expressed in muscle-rich tissues and organs (Yan et al., 2012). It had been implicated in the determination of differentiated stages of muscle cells and in myogenesis (Chen et al., 2006).

MiR-184-3p was the most highly expressed miRNA in *Cx. quinquefasciatus* described to date, and had been identified in over 39 organisms, although it has no defined role in insects (Skalsky et al., 2010). MiR-92 was significantly up-regulated miRNA in WNV-infected *Cx. quinquefasciatus* and may play a role in mediating flavivirus infection in mosquito host (Skalsky et al., 2010). Cpp-miR-71 was highly conserved in the miRNA encoding region (He et al., 2008). In *Drosophila*, the miR-13 and miR-2 family shared target genes, targeting pro-apoptotic genes and conserving target properties in distant species (Stark et al., 2003). Cpp-miR-71 which clustered with cpp-miR-13 and cpp-miR-2-3p (de Souza Gomes et al., 2013), had higher expression levels in most stages in DS-strains compared with DR1-strain. In *Caenorhabditis elegans*, miR-71 was required for life span extension (Boulias and Horvitz, 2012). Taken together, cpp-miR-71 may be involved in the control of apoptosis resulting in insecticides resistance and warrants further further investigation.

3.4. Target prediction

MiRNA works through imperfect base-pairing to target seed region sites in the 3'UTR of mRNAs resulting in translational inhibition or mRNA degradation. Using the CYP450 sequence from *Cx. quinquefasciatus*, we predicted the target gene of cpp-miR-71 using miRNA seed regions binding to the 3'UTR of mRNAs. We found the 3'UTR sequence of *CYP325BG3* contained a target site for cpp-miR-71 (Fig. 3A). We confirmed the expression level of *CYP325BG3* using qRT-PCR and found that *CYP325BG3* was up-regulated in DR2-strain (Fig. 4).

To verify that cpp-miR-71 could regulate *CYP325BG3* by directly binding to the 3'UTR of the gene, we used a dual fluorescent reporter system. The miRNA mimic worked as endogenously expressed miRNA. The PGL4.74 plasmid served as an internal control for transfection efficiency using FuGENE® HD Transfection Reagent (Mottahedin et al., 2013). *CYP325BG3* targeting by cpp-miR-71 resulted in *CYP325BG3* degradation. As predicted, the cpp-miR-71 mimic decreased luciferase activity by approximately 23% in the *CYP325BG3* UTR PMIR-reporter vector, compared to the negative mimic control (Fig. 3B). This result indicated that cpp-miR-71 can directly regulate *CYP325BG3* *in vitro*. To confirm whether cpp-miR-71 specifically inhibited *CYP325BG3* by binding to the seed sequence, the mutated reporter of the cpp-miR-71 binding site was constructed. Overexpression of cpp-miR-71 did not affect the *CYP325BG3* MUT PMIR-report vector activity (Fig. 3B). These results confirmed that *CYP325BG3* was a direct target of cpp-miR-71 with the specific binding site at the seed sequence.

3.5. Effects of cpp-miR-71 in female mosquito

To determine whether the cpp-miR-71 mimic could knockdown the target gene *in vivo*, the cpp-miR-71 mimic was injected into female adults from the DR2-strain. Compared to the control group, mosquitoes injected with the cpp-miR-71 mimic had much higher expression levels of miR-71 (Fig. 5A) and lower levels of *CYP325BG3* (Fig. 5B). These results indicated that overexpression of cpp-miR-71 would cause the down-regulation (almost 57%) of *CYP325BG3* in female mosquitoes compared with control group.

The American CDC Bottle Bioassay was applied after cpp-miR-71 injection to illustrate its contribution to pyrethroid resistance (Fig. 5C). The results showed that cpp-miR-71 injected group had a much higher mortality rate than other groups at each diagnostic time point. At 45 minutes, the mortality rate of the cpp-miR-71 injected group was 33.97%, while the NC group was 20.92% and the DEPC group was 17.24%. There was a statistically significant difference between the cpp-miR-71 injected group and the non cpp-miR-71 injected groups. At 120 minutes, the mortality of the cpp-miR-71 injected group was up to 74.13%. The control group injected with cpp-miR-71 exposed to acetone was all alive after two hours. The results showed that overexpression of cpp-miR-71 in female adults from the DR2-strain decreased resistance to deltamethrin.

Cpp-miR-71 was identical to cel-miR-71, it has been reported that cel-miR-71 may function in pathways that impacted the life span of *C.elegans* (de Lencastre et al., 2010). Cel-miR-71 increased during the aging process and promoted longevity. Moreover, research showed that

cel-miR-71 interacted with the DNA damage response pathway. In our study, cpp-miR-71 was up-regulated in the DS-strain and down-regulated in the DR-strain. The CDC Bottle Bioassay showed that cpp-miR-71 played a negative role in promoting pyrethroid resistance. However, the mechanism of cpp-miR-71 in deltamethrin resistance needs to be further investigation.

3.6. Silencing of *CYP325BG3* *in vivo*

RNA interference (RNAi) is a genetic tool that allows researchers to rapidly modulate genes of interest (Song et al., 2010). We tested the function of *CYP325BG3* *in vivo* by injecting specific siRNA into female adults. qRT-PCR was used to quantify the expression level of *CYP325BG3* relative to the control group (Fig. 6A). The group injected with siRNA showed a reduction in *CYP325BG3* expression of 57% compared to the control group. Based on the American CDC Bottle Assay, the group injected with siCYP325BG3 had a higher mortality rate than the other two groups (Fig. 6B). The control group was injected with siCYP325BG3 exposed to acetone. The results showed that a lack of *CYP325BG3* made female adults more sensitized to deltamethrin.

CYP325BG3 belongs to the CYP4 clade (Nelson, 2011). P450 enzymes from each clades have been shown to be associated with resistance to insecticides (Feyereisen, 2006). Knockdown of *CYP325BG3* made female adults more sensitive to deltamethrin. The results demonstrated that *CYP325BG3* contributed to deltamethrin resistance in mosquitoes.

4. Conclusion

In summary, we identified 100 distinct conserved miRNAs and 42 novel miRNAs from mixed growth stages (fourth-instars larvae, pupae, male and female adults) of *Cx. pipiens*. Our study provides the first large-scale miRNAs profiling of *Cx. pipiens* and identifies the precursors of these miRNAs. We also compared miRNA expression differences in DS and DR-strains and validated the differences in expression by qRT-PCR. These differentially expressed miRNAs gives us clues to further study the mechanisms of pyrethroid resistance. Cpp-miR-71 is down-regulated at most life stages in DR-strains, especially in female adult mosquitoes. MiRNAs regulate target genes by binding to their 3'UTR and we find that cpp-miR-71 could directly regulate *CYP325BG3*. This is the first study to show the important role cpp-miR-71 plays in pyrethroid resistance. In addition, our study is the first to provide evidence that the target gene *CYP325BG3* participates in pyrethroid resistance. The signaling pathway involving cpp-miR-71 and *CYP325BG3*, however, requires further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DS-strain	deltamethrin-sensitive strain
DR-strain	deltamethrin-resistant strain
Cpp-	<i>Culex pipiens-</i>

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Highlights

- We identified 100 conserved miRNAs and 42 novel miRNAs in *Culex pipiens*
- Expression of 28 miRNAs differed in deltamethrin-sensitive and -resistant strains
- Cpp-miR-71 mediates pyrethroid resistance via *cytochrome P450 325BG3* regulation
- Overexpression of cpp-miR-71 in female mosquitoes reduced resistance to deltamethrin

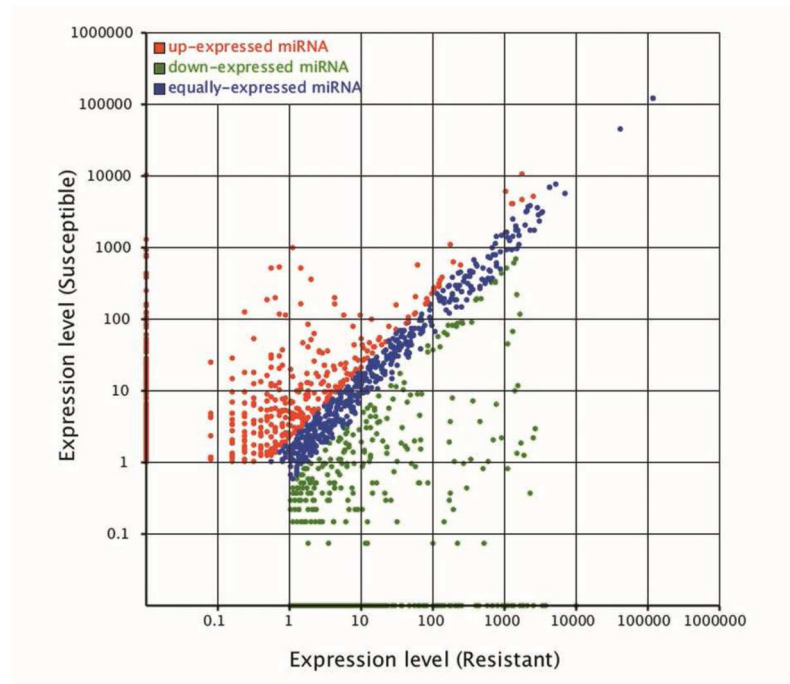


Figure 1. Scatter plot of differentially expressed miRNA in the DS-strain and DR1-strain
 Each plot represents a miRNA. Red points indicate a ratio >2 . Blue points indicate a $1/2 < \text{ratio} < 2$. Green points indicate a ratio $< 1/2$. Ratio: standard expression level in DR1-strain/ standard expression level in DS-strain.

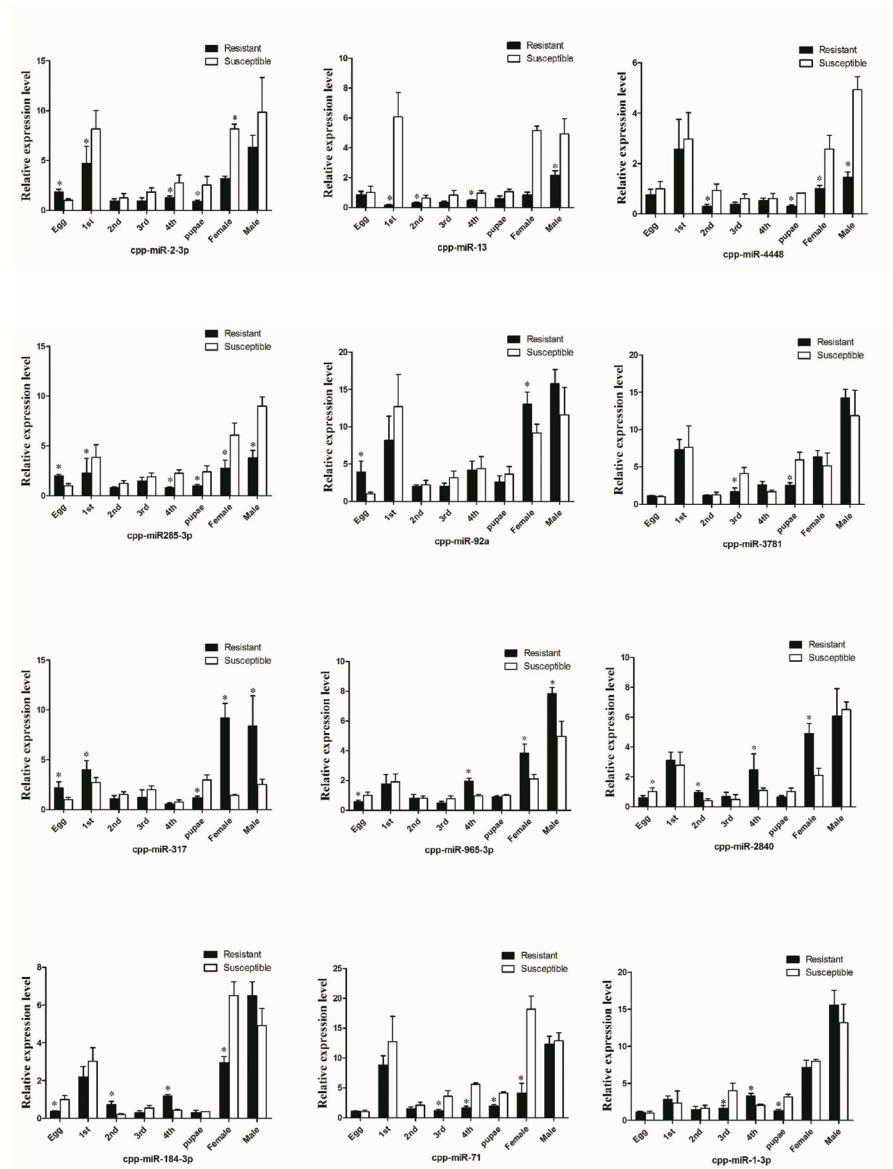


Figure 2. The expression levels of miRNAs in the DS-strain and DR1-strain measured by stem-loop RT-PCR

Expression levels of cpp-miR-2-3p, cpp-miR-13, cpp-miR-285-3p, cpp-miR-71 and cpp-miR-4448 were up-regulated in the DS-strain, while cpp-miR-965-3p, cpp-miR-317 and cpp-miR-2840 were up-regulated in the DR1-strain at most stages. The results were presented as mean± standard error (SE) of three independent experiments (* $p < 0.05$).

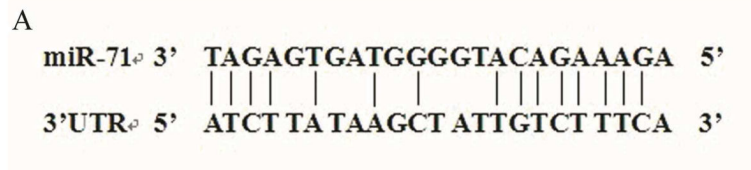


Figure 3a

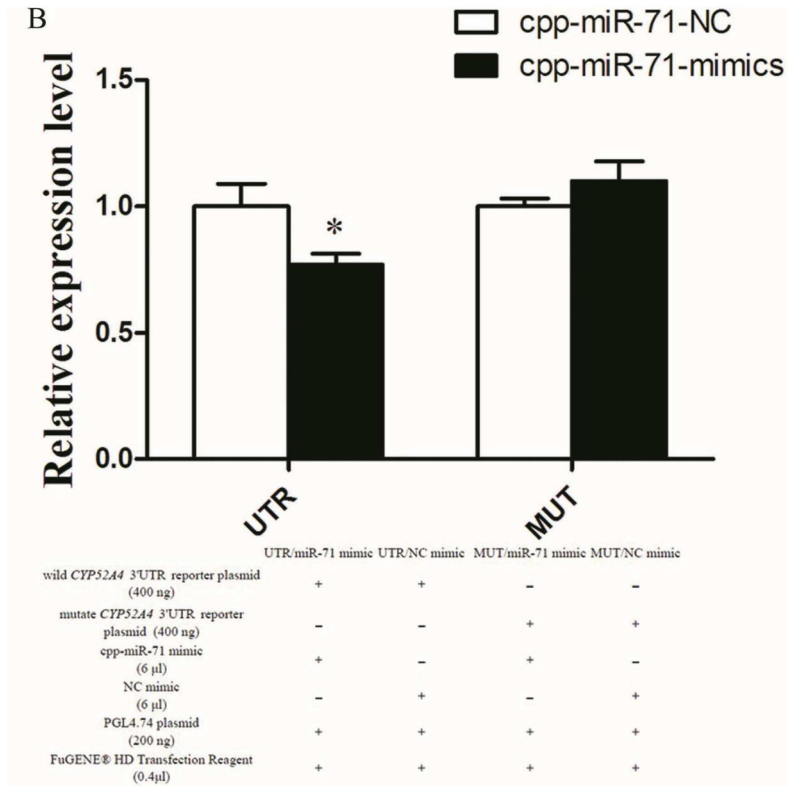


Figure 3b

Figure 3. Interaction between cpp-miR-71 and *CYP325BG3* using a dual fluorescent reporter system

(A) Base pairing between the seed sequence and the 3'UTR of *CYP325BG3*. (B) Luciferase activity decreased 23% compared to the control after overexpression of cpp-miR-71. The results were presented as mean± standard error (SE) of three independent experiments (* $p < 0.05$).

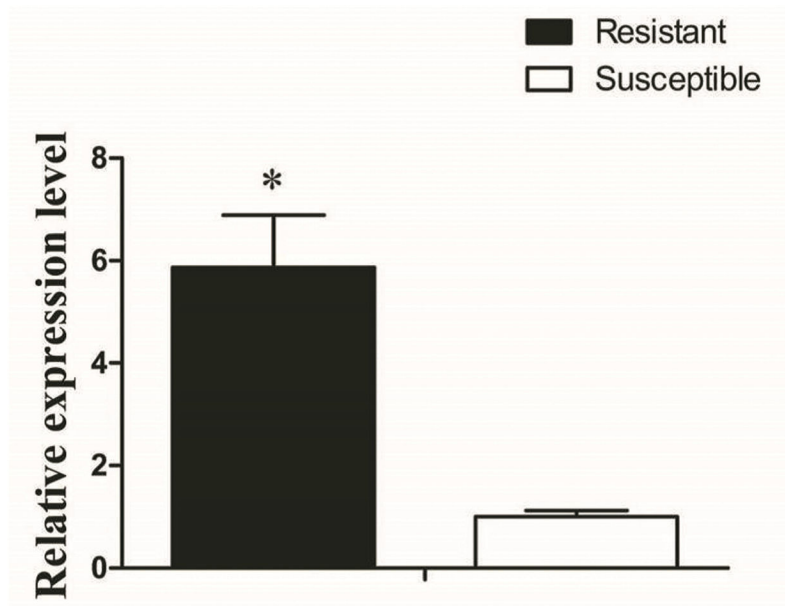


Figure 4. Expression level of *CYP325BG3* in the DR2-strain
The results were representative of three independent experiments (* $p < 0.05$).

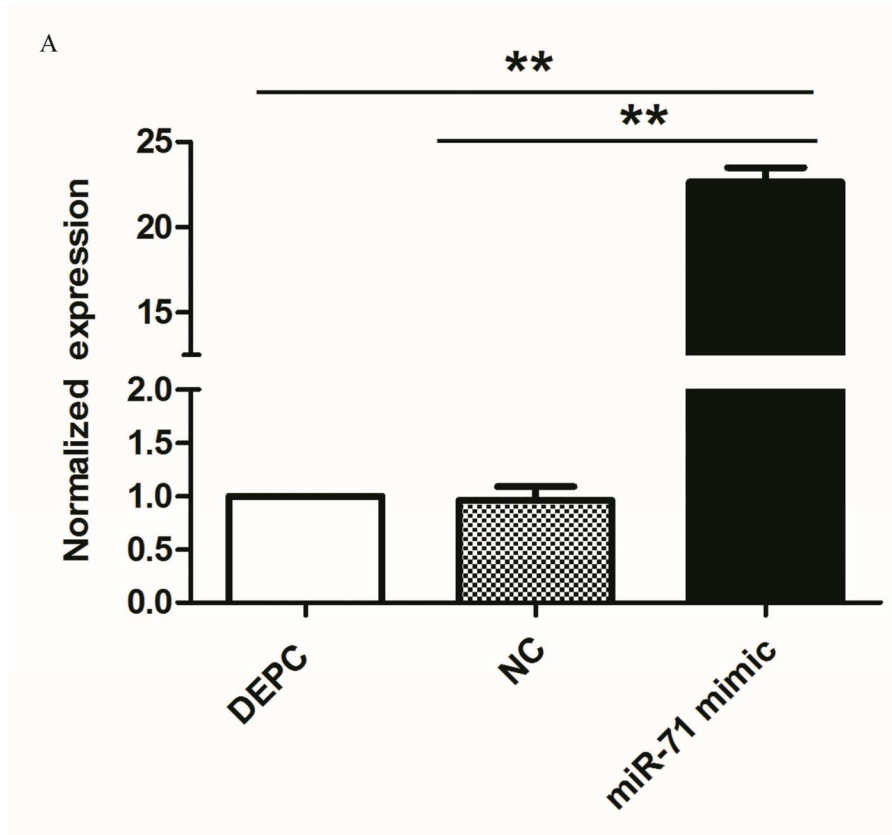


Figure 5a

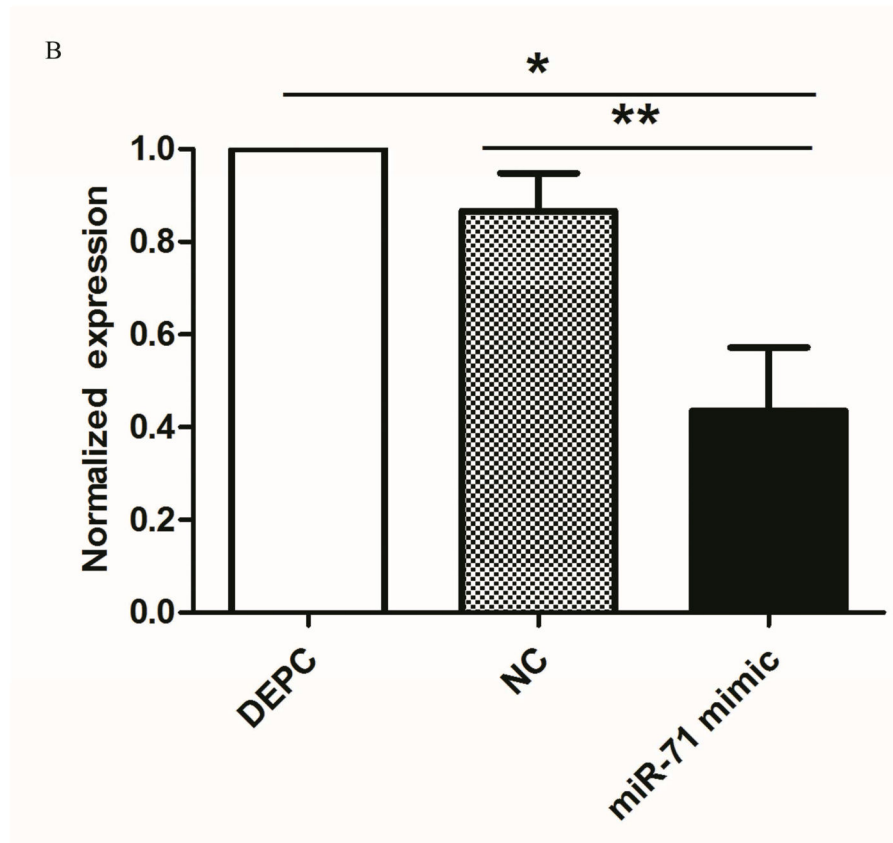


Figure 5b

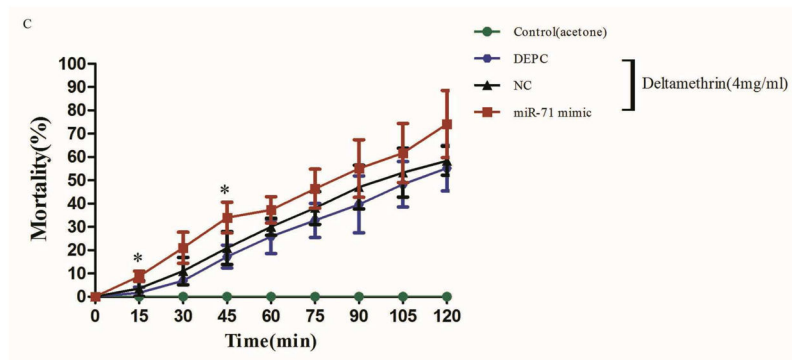


Figure 5c

Figure 5. Microinjection of the cpp-miR-71 mimic in female adult mosquitoes

(A) The expression of cpp-miR-71 as analysed by stem-loop quantitative RT-PCR after microinjection the negative control mimic and cpp-miR-71 mimic and DEPC. In the cpp-miR-71 mimic microinjection group, the expression level was 22.63 fold higher than with the DEPC microinjection. (B) qRT-PCR analysis of *CYP325BG3* after microinjection. Compared with DEPC-water group, the target gene *CYP325BG3* was knocked down almost 57% with the cpp-miR-71 microinjection. (C) Mortality of microinjected mosquitoes observed after a two hour exposure to CDC bottles treated with deltamethrin (4 mg/ml). The

results were presented as mean± standard error (SE) of three independent experiments (* $p<0.05$, ** $p<0.01$). There was no statistically significant difference between the DEPC and negative control (NC) mimic microinjection group.

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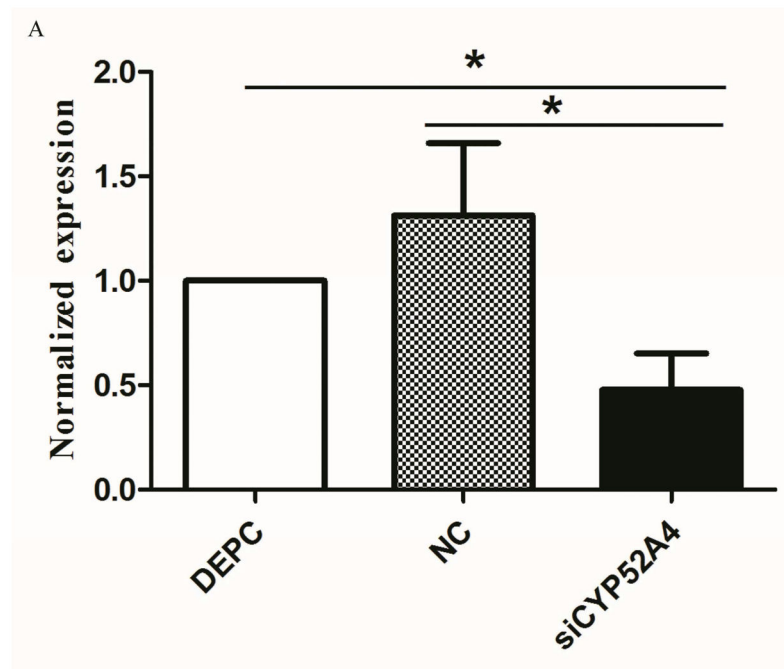


Figure 6a

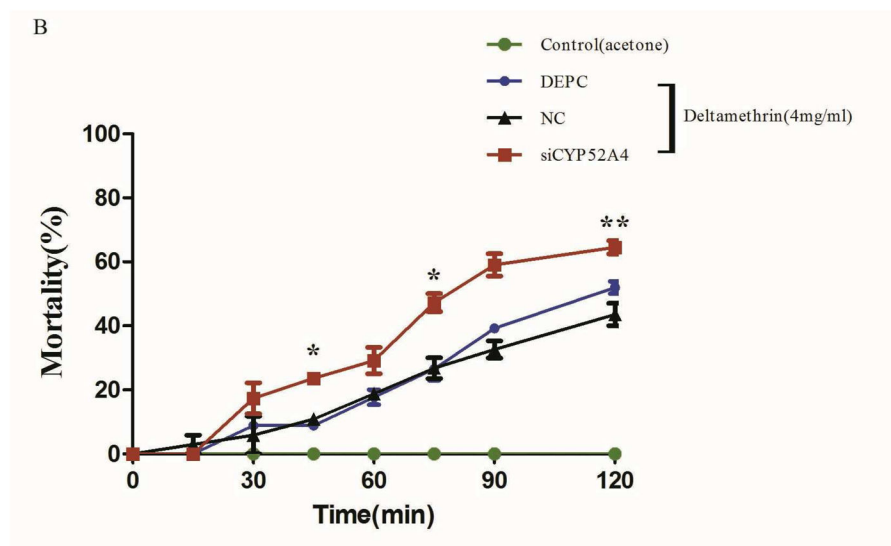


Figure 6b

Figure 6. Silencing of *CYP325BG3* in female mosquitoes

(A) qRT-PCR analysis of the knockdown efficiency of *CYP325BG3* in female mosquitoes. Compared with the DEPC-water group, the target gene *CYP325BG3* was knocked down almost 60% in the treated group. (B) Mortality of microinjected mosquitoes observed following a two hour exposure to CDC bottles treated with deltamethrin (4 mg/ml). The results are presented as mean \pm standard error (SE) of three independent experiments

(* $p < 0.05$, ** $p < 0.01$). There was no statistically significant difference between the DEPC and NC mimic microinjection groups.

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Table 1Information on the *Cx. pipiens* miRNA examined in this study

miRBase Name	Sequence	Location in genome
Previously known miRNAs that are also found in <i>Culex quinquefasciatus</i>		
cpp-bantam	UGAGGUAGUUGGUUGUAUAGU	supercont3.65: 199694-199763:-
cpp-let-7	UGAGGUAGUUGGUUGUAUAGU	supercont3.4:280600:280681:+
cpp-miR-1-3p	UGGAAUGUAAAGAAGUAUGGAG	supercont3.78:246202:246281:+
cpp-miR-2-3p-1	UAUCACAGCCAGCUUUGAAGAGC	supercont3.366:116579:116662: -
cpp-miR-2-3p-2	UAUCACAGCCAGCUUUGAAGAGC	supercont3.366:116853:116935: -
cpp-miR-2-3p-3	UAUCACAGCCAGCUUUGAAGAGC	supercont3.366:117351:117432: -
cpp-miR-7	UGGAAGACUAGUGAUUUUGUUGU	supercont3.1:3357343:3357422: -
cpp-miR-8	UAAUACUGUCAGGUAAGAUGUC	supercont3.40:815854:815935: -
cpp-miR-9	UCUUUGGUUAUCUAGCUGUA	supercont3.1009:114890:114972: -
cpp-miR-10-3p	CAAAUUCGGUUCUAGAGAGGUUU	supercont3.12:95990:96072: -
cpp-miR-11-3p	CAUCACAGUCUGAGUUCUUGCU	supercont3.153:639659:639743: -
cpp-miR-12-3p	UGAGUAUUAUCAUCAGGUACUGGU	supercont3.153: 639666-639741: -
cpp-miR-13	UAUCACAGCCAUUUUGACGAGUU	supercont3.366:116983:117072: -
cpp-miR-14-3p	UCAGUCUUUUUCUCUCUCCUAU	supercont3.676:52241:52327: -
cpp-miR-31	UGGCAAGAUGUUGGCAUAGCUGA	supercont3.559:256532:256609: -
cpp-miR-33	GUGCAUUGUAGUUGCAUUGCA	supercont3.1258: 69328-69403: -
cpp-miR-71	AGAAAGACAUGGGUAGUGAGAU	supercont3.366:117493:117583: -
cpp-miR-79-3p	UAAAGCUAGAUUACCAAAGCAU	supercont3.83:80579:80662:+
cpp-miR-87	GUGAGCAAUUUUCAGGUGUGU	supercont3.431:379743:379819:+
cpp-miR-92a	UAUUGCACUUGUCCCGCCUAU	supercont3.722:174902:174982: -
cpp-miR-92b	AAUUGCACUUGUCCCGCCUGC	supercont3.722:164904:164984: -
cpp-miR-100	AACCCGUAGAUCCGAACUUGUG	supercont3.4:271404:271484:+
cpp-miR-124	UAAGGCACGCGGUGAAUGCCAA	supercont3.8:2074719:2074803:+
cpp-miR-125	UCCUGAGACCCUAAUUGUGA	supercont3.4:280965:281043:+
cpp-miR-133-3p	UUGGUCCCUUCAACCAGCUGU	supercont3.1189: 55672-55785:+
cpp-miR-137-3p	UAUUGCUUGAGAAUACACGUAG	supercont3.1714:27556:27636: -
cpp-miR-184-3p	UGGACGGAGAACUGAUAAAGGC	supercont3.567:240302:240382: -
cpp-miR-190-1	AGAU AUGUUGAU AUUCUUGGUUG	supercont3.181:347902:347986: -
cpp-miR-190-2	AGAU AUGUUGAU AUUCUUGGUUG	supercont3.1806:19610:19690: -
cpp-miR-210-3p	CUUGUGCGUGUGACAACGGCUAU	supercont3.549:157647:157724: -
cpp-miR-252-1	CUAAGUACUAGUGCCGCAGGAG	supercont3.1787:6793:6867: -
cpp-miR-252-2	CUAAGUACUAGUGCCGCAGGAG	supercont3.174:422878:422969: -
cpp-miR-263	AATGGCACTGGAAGAATTCACGG	supercont3.219:351799:351880: -
cpp-miR-275	TCAGGTACCTGAAGTAGCGCG	supercont3.291:329765:329845:+
cpp-miR-276-3p-1	UAGGAACUUCUACCGUGCUCU	supercont3.136:340857:340942:+
cpp-miR-276-3p-2	UAGGAACUUCUACCGUGCUCU	supercont3.136:541142:541223:+
cpp-miR-276-3p-3	UAGGAACUUCUACCGUGCUCU	supercont3.2457:876:961:+
cpp-miR-277	UAAAUGCACUAUCUGGUACGACA	supercont3.36:1153726:1153817:+

miRBase Name	Sequence	Location in genome
cpp-miR-278-3p	UCGGUGGGACUUUCGUCCGUUU	supercont3.16:1026152:1026243:+
cpp-miR-279-3p	UGACUAGAUCACACUCAUUA	supercont3.19:1441114:1441197: -
cpp-miR-281-1	AAGAGAGCUAUCCGUCGACAGU	supercont3.1661:13247:13325:+
cpp-miR-281-2	AAGAGAGCUAUCCGUCGACAGU	supercont3.640:99734:99812:+
cpp-miR-283	CAAUAUCAGCUGGUAUUCUGGG	supercont3.57: 559440-559549 :+
cpp-miR-285-3p	UAGCACCAUUCGAAAUCAGUAC	supercont3.98:262280:262362: -
cpp-miR-305	ATTGTACTTCATCAGGTGCTC	supercont3.291:339122:339204:+
cpp-miR-306	UCAGGUACUGAGUGACUCUCAG	supercont3.83: 80431-80502:+
cpp-miR-308	CGCGGTATATTCTTGTGGCTTG	supercont3.98:764123:764204:+
cpp-miR-309-3p	UCACUGGGCAUAGUUUGUCGC	supercont3.145: 66026-66116: -
cpp-miR-315	UUUUGAUUGUUCUCAGAAAGC	supercont3.438:61916:61999:+
cpp-miR-316	UGUCUUUUUCCGCUACUGCCG	supercont3.496:152458:152539: -
cpp-miR-317-3p-1	UGAACACAGCUGGUGGUAUCU	supercont3.36:1133161:1133241:+
cpp-miR-317-3p-2	UGAACACAGCUGGUGGUAUCU	supercont3.36:1134827:1134907:+
cpp-miR-375-1	UUUGUUCGUUUGGCUCGAGUUA	supercont3.4:25124:25212: -
cpp-miR-375-2	UUUGUUCGUUUGGCUCGAGUUA	supercont3.455:42526:42614:+
cpp-miR-932-3p	UGCAAGCAAUGUGGAAGUGA	supercont3.261:301401:301481: -
cpp-miR-957-3p	UGAAACCGUCCAAAACUGAGGC	supercont3.787:29537:29622:+
cpp-miR-965-3p	UAAGCGUAUAGCUUUUCCCAU	supercont3.48:484122:484208:+
cpp-miR-970-3p	UCAUAAGACACACGCGCUAU	supercont3.495: 35917-36003:+
cpp-miR-980-3p	TAGCTGCCTTGTGAAGGGCTTA	supercont3.263:352870:352954:+
cpp-miR-981	UUCGUUGUCGACGAAACCUGCA	supercont3.431: 151310-151408:+
cpp-miR-988-3p	CCCUUGUUGCAAACCUCACGC	supercont3.791:14282:14361:+
cpp-miR-989-3p	UGUGAUGUGACGUAGUGGUAC	supercont3.315:321306:321394:+
cpp-miR-993	UACCCUGUAGUUCGGGCUUUU	supercont3.12:55477:55565:+
cpp-miR-996	UGACUAGAUACAUGCUCGU	supercont3.19:1437000:1437085: -
cpp-miR-998	ACTGAATTCTCGTGGGTCTGCA	supercont3.153:639515:639597: -
cpp-miR-999-3p	UGUUAACUGUAAGACUGUGUCU	supercont3.14:96865:96948:+
cpp-miR-1000	AUAUUGUCCUGUCACAGCAGU	supercont3.153: 102798-102891: -
cpp-miR-1174	CUGGGUAUUUAGAUAUCGGC	supercont3.86:865891:865964:+
cpp-miR-1175	AAGUGGAGUAGUGGUCUCAUCG	supercont3.86:866106:866187:+
cpp-miR-1889	UAAUCUAAAUUGUAACAGUGG	supercont3.57: 562539-562677:+
cpp-miR-1890	UGAAAUCUUUGAUUAGGUCU	supercont3.64:982778:982862: -
cpp-miR-1891	UGAGGAGUUAUUUGCGUGUUU	supercont3.829: 180333-180418: -
cpp-miR-2941-3p-1	UAGUACGGCUAGAACUCCACGG	supercont3.5:753632:753715: -
cpp-miR-2941-3p-2	UAGUACGGCUAGAACUCCACGG	supercont3.5:753786:753871: -
cpp-miR-2951-1	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 4408-4477:+
cpp-miR-2951-2	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 13130-13199:+
cpp-miR-2951-3	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 21931-22000 :+
cpp-miR-2951-4	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 30698-30767:+
cpp-miR-2951-5	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 39499-39568:+
cpp-miR-2951-6	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464: 48275-48344:+

miRBase Name	Sequence	Location in genome
cpp-miR-2951-7	GAAGAGCTCAGCACGCAGGGGTG	supercont3.1464:56986-57055:+
cpp-miR-2951-8	GAAGAGCTCAGCACGCAGGGGTG	supercont3.679:54985-55054:+
cpp-miR-2952-3p	UAGUACGGCCAUGACUGAGGGC	supercont3.5:753920-753983:–
cpp-miR-iab-4	ACGUAUACUGAAUGUAUCCUGA	supercont3.12:681153:681228:+
Previously known miRNAs that are also found in non-<i>Culex quinquefasciatus</i> species		
cpp-miR-2779-1	AUAUCCGGCUCGAAGGACCA	supercont3.1527:60074:60167:+
cpp-miR-2779-2	AUAUCCGGCUCGAAGGACCA	supercont3.2199:21971:22064:–
cpp-miR-2779-3	AUAUCCGGCUCGAAGGACCA	supercont3.806:109239:109311:–
cpp-miR-2796-3p	GUAGGCCGCGGAAACUACUUGC	supercont3.1234:55494:55572:–
cpp-miR-2840	UAGGAACUGGAAGAAGAGGAGG	supercont3.830:78060:78155:–
cpp-miR-2942	UAUUCGAGACUUCACGAGUAAU	supercont3.277:323072:323166:+
cpp-miR-2944b	GAAGGAACUCCCGGUGUGAUUA	supercont3.145:66345:66420:–
cpp-miR-2945	UGACUAGAGGCAGACUCGUUUA	supercont3.4:184409:184491:+
cpp-miR-2981	CCGGGCCGGCGGGCGGG	supercont3.772:7842:7925:–
cpp-miR-3781	UAAGUGAUUGAUCGAUCGUGGAU	supercont3.1005:38090:38187:–
cpp-miR-4682	UCUGAGUUCUGGAGCCUGGUCU	supercont3.1:1608156:1608242:+
cpp-miR-4448	GGCUCCUUGGUCUAGGGGUA	supercont3.461:292111:292200:–
cpp-miR-307	UCACAACCUCCUUGAGUGAGCGA	supercont3.16:157813:157891:–
cpp-miR-493-3p	UGAAGGUCCUACUGUGGCCAGG	supercont3.147:509902:509990:+
cpp-miR-929-3p	CUCCCUAACGGAGUCAGAUUG	supercont3.60:858168:858245:–
cpp-miR-1290	UGGAUUUUUGGAUCAGAGA	supercont3.383:279453:279536:+
Novel miRNAs found in this study		
cpp-novel-miR1	AGAGCTAATTGGAGACTTCTTG	supercont3.64:982778:982859:–
cpp-novel-miR2-1	GTGTCCTGTCACGGTCGCCA	supercont3.360:171826:171903:–
cpp-novel-miR2-2	GTGTCCTGTCACGGTCGCCA	supercont3.88:405605:405682:–
cpp-novel-miR3-3p	GTTTGAACCTGATCCGCGCTGA	supercont3.112:3529:3623:–
cpp-novel-miR4	GTGCTTTTCGTTGGAACCTG	supercont3.153:639486:639575:–
cpp-novel-miR5	AATTAGAAATCACACAAACGTT	supercont3.316:156915:156993:+
cpp-novel-miR6-3p	TAGGGAACAGATTGGCCAATG	supercont3.1403:25947:26028:+
cpp-novel-miR7-3p-1	ACATCGCGTGTCTGTTGGCAT	supercont3.1002:30216:30294:–
cpp-novel-miR7-3p-2	ACATCGCGTGTCTGTTGGCAT	supercont3.1002:37099:37177:–
cpp-novel-miR7-3p-3	ACATCGCGTGTCTGTTGGCAT	supercont3.1002:45236:45314:–
cpp-novel-miR7-3p-4	ACATCGCGTGTCTGTTGGCAT	supercont3.1002:54195:54273:–
cpp-novel-miR8-3p	ACACGTCCATTAACCTGTTAC	supercont3.829:180337:180416:–
cpp-novel-miR9-1	AATCGGAATTCTAAAACGGAA	supercont3.156:169435:169530:–
cpp-novel-miR9-2	AATCGGAATTCTAAAACGGAA	supercont3.213:211573:211659:–
cpp-novel-miR9-3	AATCGGAATTCTAAAACGGAA	supercont3.664:19971:20057:+
cpp-novel-miR9-4	AATCGGAATTCTAAAACGGAA	supercont3.924:122727:122815:+
cpp-novel-miR10	TGATCTTGATTTTGTATGCTCC	supercont3.329:155673:155749:–
cpp-novel-miR11-3p	CAGTGCATGGCCAACACGGTTT	supercont3.1328:35831:35912:+
cpp-novel-miR12	GGTGTTCACCTGCCGCTGTATG	supercont3.8:2074722:2074801:+
cpp-novel-miR13	GTTAGTTTTGGCGGGTTTTAGT	supercont3.787:29538:29623:+

miRBase Name	Sequence	Location in genome
cpp-novel-miR14-3p	GGCGCGAGCGTGTGTTATTC	supercont3.196:659156:659228:+
cpp-novel-miR15	ATTTGTGGTATATGTCGGACGAG	supercont3.829:126138:126232:+
cpp-novel-miR16-3p	AAGGAGTGGAACCTGGTCGCGGA	supercont3.112:109706:109789:+
cpp-novel-miR17-3p	CGGGATTCCAACCTGATATCCAC	supercont3.57:559449:559540:+
cpp-novel-miR18-3p-1	TTGCAGTGGATGGTCGTTTGACG	supercont3.1369:75814:75893: -
cpp-novel-miR18-3p-2	TTGCAGTGGATGGTCGTTTGACG	supercont3.91:950407:950490: -
cpp-novel-miR19-3p-1	CAGGAGTTGATTTGGAGGACACCA	supercont3.1422:16129:16229:+
cpp-novel-miR19-3p-2	CAGGAGTTGATTTGGAGGACACCA	supercont3.830:159415:159515: -
cpp-novel-miR20-1	TGATTGTTTACTCGATCGTTGG	supercont3.533:194362:194441:+
cpp-novel-miR20-2	TGATTGTTTACTCGATCGTTGG	supercont3.816:142480:142580:+
cpp-novel-miR20-3	TGATTGTTTACTCGATCGTTGG	supercont3.843:44076:44155:+
cpp-novel-miR21-1	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.1761:8353:8427: -
cpp-novel-miR21-2	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.1761:10406:10480: -
cpp-novel-miR21-3	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.207:246904:246978: -
cpp-novel-miR21-4	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.207:248944:249018: -
cpp-novel-miR21-5	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.2777:15561:15635: -
cpp-novel-miR21-6	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.46:879159:879234:+
cpp-novel-miR21-7	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.65:252545:252619:+
cpp-novel-miR21-8	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.65:254717:254791:+
cpp-novel-miR21-9	TTGTCAGTGACGGGTAGTTAGGTT	upercont3.69:203697:203771: -
cpp-novel-miR21-10	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.69:205631:205705: -
cpp-novel-miR21-11	TTGTCAGTGACGGGTAGTTAGGTT	supercont3.78:292181:292255: -

Table 2

Differential expression of miRNAs in *Culex pipiens* fold-change

	Susceptible-expressed	Resistant-expressed	Susceptible-std	Resistant-std	(log2 Susceptible/Resistant)	p-value
cpp-bantam	3330	3181	242.0006	257.4568	-0.08931975	0.012522392
cpp-let-7	23296	19387	1692.9868	1569.1023	0.10963131	5.10E-15
cpp-miR-1-3p	1670635	1467552	121409.8156	118777.4939	0.03162357	1.16E-83
cpp-miR-2-3p-1	4293	1532	311.9846	123.9936	1.33120916	2.27E-93
cpp-miR-7	856	614	62.208	49.6946	0.32401103	2.00E-05
cpp-miR-8	26618	17744	1934.4061	1436.1248	0.42970957	8.75E-211
cpp-miR-9	5397	3660	392.2154	296.225	0.40495278	8.29E-40
cpp-miR-10-3p	3020	2353	219.472	190.4419	0.20468597	2.33E-07
cpp-miR-11-3p	625	424	45.4205	34.3168	0.40442856	7.33E-06
cpp-miR-12-3p	506	422	36.7725	34.1549	0.10653469	0.263488572
cpp-miR-13	3510	1297	255.0817	104.9737	1.28093148	2.71E-183
cpp-miR-14-3p	6029	2816	438.1446	227.9152	0.94290993	5.04E-192
cpp-miR-31	10745	9409	780.8698	761.5249	0.03619081	0.075629556
cpp-miR-33	889	464	64.6062	37.5542	0.78269836	3.46E-22
cpp-miR-71	7535	5245	547.59	424.5083	0.36730334	4.74E-46
cpp-miR-79-3p	671	383	48.7635	30.9984	0.65360793	6.62E-13
cpp-miR-87	677	308	49.1995	24.9282	0.98086494	1.49E-24
cpp-miR-92a	2238	991	162.6419	80.2074	1.01989172	2.79E-82
cpp-miR-92b	339	213	24.6361	17.2393	0.51507269	3.77E-05
cpp-miR-100	4049	2899	294.2524	234.6329	0.32664886	8.50E-21
cpp-miR-124	5419	4123	393.8142	333.6983	0.2389708	9.01E-16
cpp-miR-125	935	439	67.9491	35.5308	0.93538427	6.81E-31
cpp-miR-133-3p	5212	1670	378.7709	135.1628	1.48662735	0
cpp-miR-137-3p	119	105	8.6481	8.4983	0.02520893	0.900201288
cpp-miR-184-3p	93422	53214	6789.2435	4306.9176	0.65659511	0
cpp-miR-190-1	1319	808	95.8555	65.3961	0.55165661	4.61E-18
cpp-miR-210-3p	4563	9404	331.6062	761.1203	-1.19865352	0
cpp-miR-252-1	2677	1521	194.5452	123.1034	0.66023478	1.33E-47

	Susceptible-expressed	Resistant-expressed	Susceptible-std	Resistant-std	(log ₂ Susceptible/Resistant)	p-value
cpp-miR-263	55760	16302	4052.2384	1319.4154	1.6188202	0
cpp-miR-275	52109	28270	3786.9098	2288.055	0.72689933	0
cpp-miR-276-3p-1	6372	4239	463.0714	343.0869	0.43266062	1.53E-52
cpp-miR-277	2614	1779	189.9668	143.9848	0.39983079	1.08E-19
cpp-miR-278-3p	52	21	3.779	1.6997	1.15272439	0.001378883
cpp-miR-279-3p	2658	4041	215.1274	293.671	0.44900952	3.08E-36
cpp-miR-281-1	340	181	24.7088		0.75418337	6.84E-09
cpp-miR-283	1795	1929	130.4478	156.1252	-0.25923081	4.22E-08
cpp-miR-285-3p	1667	717	121.1456	58.031	1.06184631	7.84E-66
cpp-miR-305	6083	4461	442.069	361.0546	0.29205455	6.13E-25
cpp-miR-306	31595	38138	2296.0989	3086.7295	-0.42689428	0
cpp-miR-308	151	155	10.9736	12.545	-0.19307558	0.241100194
cpp-miR-309-3p	25	32023	0.01	2591.8071	-17.98360325	0
cpp-miR-315	225	187	16.3514	15.135	0.11152549	0.436931367
cpp-miR-316	660	285	47.9641	23.0667	1.05614336	7.34E-27
cpp-miR-317-3p-1	33482	16531	2433.2325	1337.9497	0.8628503	0
cpp-miR-375-1	235	172	17.0781	13.921	0.29488463	0.041400308
cpp-miR-932-3p	759	507	55.1587	41.0345	0.42675108	2.05E-07
cpp-miR-957-3p	549	360	39.8974	29.1369	0.45344734	3.00E-06
cpp-miR-965-3p	11	6299	0.7994	509.8146	-9.3168394	0
cpp-miR-970-3p	4604	3366	334.5858	272.4299	0.29649117	8.93E-20
cpp-miR-980-3p	113	45	8.212	3.5612	1.17296309	1.51E-06
cpp-miR-981	749	226	54.432	18.2915	1.57328166	1.48E-54
cpp-miR-988-3p	57	18	4.1424	1.4568	1.50766404	3.81E-05
cpp-miR-989-3p	2507	4928	182.1908	411.8013	-1.13040196	5.93E-238
cpp-miR-993	1321	882	96.0008	71.3854	0.42741739	6.64E-12
cpp-miR-996	11712	10931	851.1445	884.7092	-0.05579924	0.003638387
cpp-miR-998	593	324	43.095	26.2232	0.71667675	2.38E-13
cpp-miR-999-3p	1430	1137	103.9222	92.024	0.17542181	0.002182576
cpp-miR-1000	228	92	16.5694	7.4461	1.15396446	1.36E-11
cpp-miR-1174	5216	2385	379.0616	193.0319	0.97359303	2.16E-175

	Susceptible-expressed	Resistant-expressed	Susceptible-std	Resistant-std	(log2 Susceptible/Resistant)	p-value
cpp-miR-1175	13407	8399	974.325	679.7798	0.51933559	7.76E-151
cpp-miR-1889	21	19	1.5261	1.5378	-0.01101838	0.972158615
cpp-miR-1890	234	144	17.0054	11.6548	0.54506869	0.000316285
cpp-miR-1891	2235	1136	162.4238	91.9431	0.82094983	1.15E-57
cpp-miR-2941-3p-1	1199	2750	87.1348	222.5734	-1.35296026	6.70E-177
cpp-miR-2951-1	43267	24845	3144.3364	2010.8499	0.64495018	0
cpp-miR-2952-3p	68	134	4.9418	10.8454	-1.13397473	5.48E-08
cpp-miR-iab-4	596	630	43.313	50.9896	-0.23540292	0.004279915
cpp-miR-2779-1	3907	4295	283.9328	347.6193	-0.29195866	5.17E-20
cpp-miR-2796-3p	19851	19927	1442.6289	1612.8077	-0.1608742	1.03E-28
cpp-miR-2840	2	398	0.1453	32.2124	-7.79243765	9.08E-126
cpp-miR-2942	251	104	18.2409	8.4173	1.11574747	4.92E-12
cpp-miR-2944b	20	12	1.4535	0.9712	0.58169074	0.277420799
cpp-miR-2945	38776	37021	2817.9626	2996.3242	-0.08854127	3.08E-17
cpp-miR-2981	81697	13083	5937.1543	1058.8831	2.48722828	0
cpp-miR-3781	1262	563	91.7131	45.5669	1.00914159	2.16E-46
cpp-miR-4682	2	73	0.01	5.9083	-9.2065993	1.77E-24
cpp-miR-4448	143222	21921	10408.3517	1774.1937	2.55250619	0
cpp-miR-307	56	21	4.0697	1.6997	1.25964232	0.00036701
cpp-miR-493-3p	122	872	8.8661	70.576	-2.99280613	1.06E-158
cpp-miR-929-3p	32	27	2.3255	2.1853	0.08970959	0.82070986
cpp-miR-1290	4988	7443	362.4922	602.4052	-0.73278427	1.80E-173
cpp-novel-miR1	1071	759	77.8326	61.4303	0.34142412	5.37E-07
cpp-novel-miR2-1	326	178	23.6914	14.4066	0.71763356	5.52E-08
cpp-novel-miR3-3p	115	66	8.3574	5.3418	0.64572823	0.003377852
cpp-novel-miR4	80	65	5.8138	5.2608	0.14419924	0.554453216
cpp-novel-miR5	76	54	5.5231	4.3705	0.33767991	0.190166848
cpp-novel-miR6-3p	78	38	5.6685	3.0756	0.88209913	0.001598861
cpp-novel-miR7-3p-1	416	40	30.2319	3.2374	3.22316412	1.07E-71
cpp-novel-miR8-3p	61	46	4.433	3.723	0.25181775	0.376102001
cpp-novel-miR9-1	192	144	13.9532	11.6548	0.25967178	0.102701359

	Susceptible-expressed	Resistant-expressed	Susceptible-std	Resistant-std	(log2 Susceptible/Resistant)	p-value
cpp-novel-miR10	43	32	3.1249	2.5899	0.27091363	0.427849104
cpp-novel-miR11-3p	62	11	4.5057	0.8903	2.3393878	8.04E-09
cpp-novel-miR12	36	33	2.6162	2.6709	-0.02985313	0.925384881
cpp-novel-miR13	42	21	3.0523	1.6997	0.84461664	0.026542101
cpp-novel-miR14-3p	30	22	2.1802	1.7806	0.29209703	0.479901119
cpp-novel-miR15	26	18	1.8895	1.4568	0.37520169	0.405654141
cpp-novel-miR16-3p	21	16	1.5261	1.295	0.2368974	0.633309487
cpp-novel-miR17-3p	20	15	1.4535	1.214	0.25976265	0.611351264
cpp-novel-miR18-3p-1	34	28	2.4709	2.2662	0.12476143	0.743660138
cpp-novel-miR19-3p-1	22	28	1.5988	2.2662	-0.50328571	0.220271427
cpp-novel-miR20-1	33	33	2.3982	2.6709	-0.15537398	0.658290585
cpp-novel-miR21-1	99	110	7.1946	8.9029	-0.30736087	0.123569093