

miRNA Genes of an Invasive Vector Mosquito, *Aedes albopictus*

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Abstract

Aedes albopictus, a vector of Dengue and Chikungunya viruses, is a robust invasive species in both tropical and temperate environments. MicroRNAs (miRNAs) regulate gene expression and biological processes including embryonic development, innate immunity and infection. While a number of miRNAs have been discovered in some mosquitoes, no comprehensive effort has been made to characterize them from different developmental stages from a single species. Systematic analysis of miRNAs in *Ae. albopictus* will improve our understanding of its basic biology and inform novel strategies to prevent virus transmission. Between 10–14 million Illumina sequencing reads per sample were obtained from embryos, larvae, pupae, adult males, sugar-fed and blood-fed adult females. A total of 119 miRNA genes represented by 215 miRNA or miRNA star (miRNA*) sequences were identified, 15 of which are novel. Eleven, two, and two of the newly-discovered miRNA genes appear specific to *Aedes*, *Culicinae*, and *Culicidae*, respectively. A number of miRNAs accumulate predominantly in one or two developmental stages and the large number that showed differences in abundance following a blood meal likely are important in blood-induced mosquito biology. Gene Ontology (GO) analysis of the targets of all *Ae. albopictus* miRNAs provides a useful starting point for the study of their functions in mosquitoes. This study is the first systematic analysis of miRNAs based on deep-sequencing of small RNA samples of all developmental stages of a mosquito species. A number of miRNAs are related to specific physiological states, most notably, pre- and post-blood feeding. The distribution of lineage-specific miRNAs is consistent with mosquito phylogeny and the presence of a number of *Aedes*-specific miRNAs likely reflects the divergence between the *Aedes* and *Culex* genera.

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Introduction

MicroRNAs (miRNAs) are ~22 nucleotides (nt) in length and modulate gene expression by targeting cognate mRNAs for cleavage or translational repression. miRNAs are distributed widely in metazoans and plants and are involved in the regulation of many biological processes including apoptosis, cancer, embryonic development, immunity and infection [1–3]. miRNAs also may have a role in mosquito responses to infection by malaria parasite and arboviruses [4,5]. Many miRNAs are transcribed from independent promoters to generate the primary miRNAs (pri-miRNAs), which could contain one or more regions of complementary bases that form secondary structures (hairpins). These hairpins are recognized and liberated by the Microprocessor complex to make precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytosol and processed further by Dicer to make the ~22-nt miRNA:miRNA* duplex. The duplex is separated by a helicase and the single-stranded miRNA is loaded into the RISC complex. The miRNA* strand is normally rapidly

degraded [6]. Other miRNAs reside in introns of genes and their biogenesis is independent of the Microprocessor complex [7,8].

Deep-sequencing coupled with bioinformatic analyses has produced comprehensive catalogs of miRNAs in model organisms. For example, there are 171 miRNAs in *Drosophila melanogaster* discovered in a large number of samples derived from different developmental stages and tissues and from analysis of 12 *Drosophila* genomes [9,10,11]. Deep-sequencing efforts to uncover miRNAs in mosquitoes have been limited to specific developmental stages or cell lines and the number discovered is relatively low compared to *D. melanogaster*. A small number of miRNAs were verified experimentally in two *Anopheles* mosquito species [4,12]. Ninety-eight miRNA genes that produce 86 distinct miRNAs were discovered in *Aedes aegypti*, mostly by small-scale 454 sequencing of embryo and midgut samples [13]. A total of 65 and 77 miRNAs were discovered following Illumina sequencing of small RNAs from an *Ae. albopictus* cultured cell line and blood-fed *Culex quinquefasciatus* females, respectively [5]. While most of these

miRNAs are conserved across divergent species, 11 distinct miRNA genes are found only in mosquitoes, some of which are restricted to certain taxonomic groups [5,13].

Here we report discovery of 119 miRNA genes through deep-sequencing of small RNAs isolated from multiple developmental stages of *Ae. albopictus*, of which 15 are novel and appear to be only in mosquitoes. We chose *Ae. albopictus* for this study because of its rapid expansion in world-wide distribution and its emerging importance as a vector for Dengue and Chikungunya viruses [14]. Our analysis has doubled the number of known mosquito-specific miRNAs, uncovered miRNAs showing stage-specific and blood-meal-regulated expression profiles, and provided the basis for the investigations of the function and evolution of mosquito miRNAs.

Materials and Methods

Ethics Statement

All vertebrate animals were housed and handled in strict accordance with the guidelines of the institutional and national Committees of Animal Use and Protection. All experimental procedures on mice were approved by the Committee on the

Ethics of Animal Experiments of Southern Medical University (Permit Number: SCXK 2006-0015).

Mosquitoes

The CDC (Guangdong, China) strain of *Aedes albopictus* originated in Guangzhou, Guangdong province, PRC, and was established in the laboratory in 1981. All mosquitoes were maintained in humidified incubators at $25 \pm 1^\circ\text{C}$ on a 12 hour light:dark cycle.

Aedes albopictus Sample Preparation for Illumina Sequencing

Embryos were collected 0–24 hours after egg deposition by placing a damp collection cup within a cage. Larval samples were collected at each instar and combined. Pupal samples were collected from a pool of varied ages. Male and female adults were collected five days post-emergence. Three- to five-day old adult females were fed on mouse blood and collected at 1, 3 and 5 days after feeding and pooled. Total RNA was isolated using Trizol (Invitrogen). Approximately 20 μg of the total RNAs were

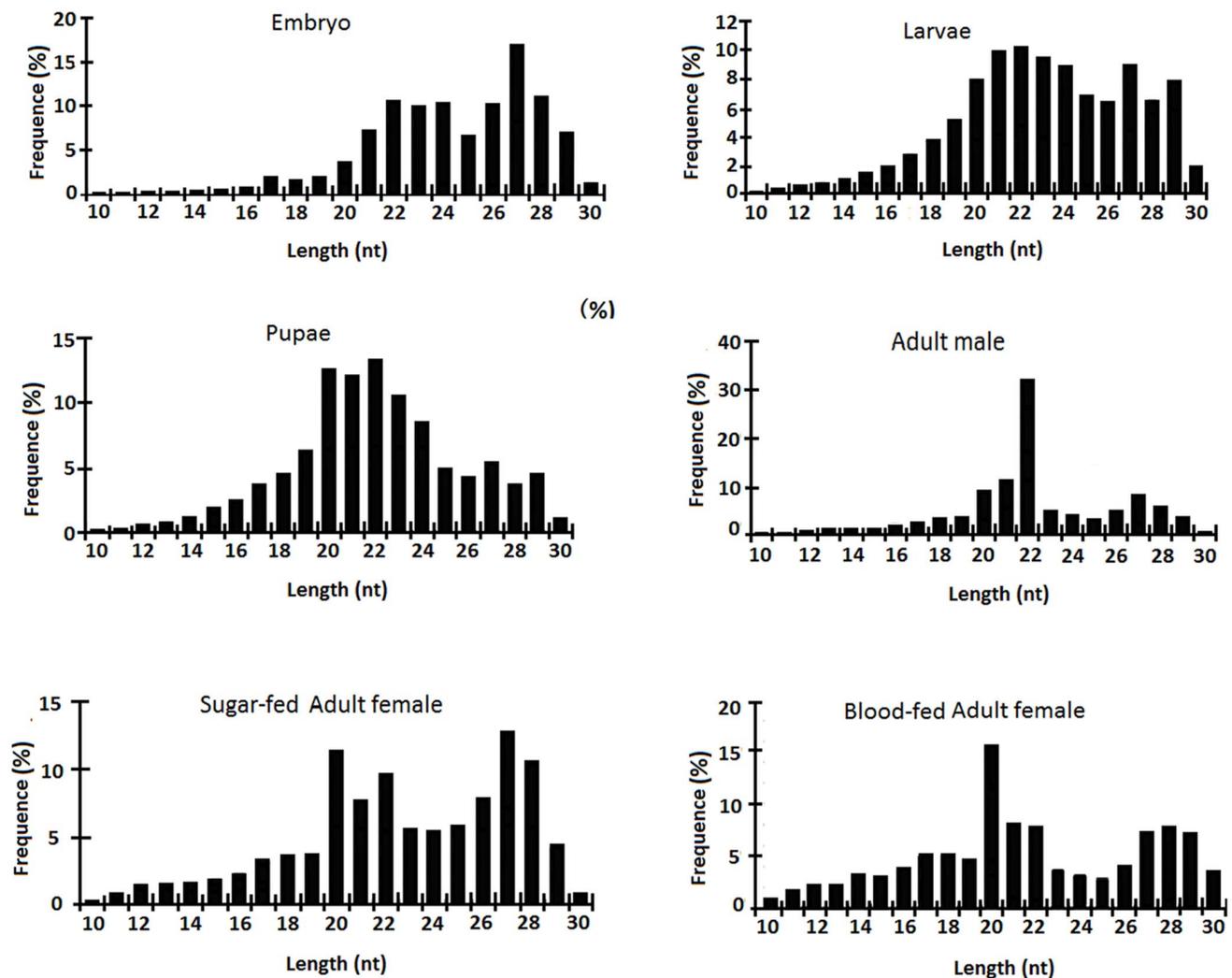


Figure 1. Size distribution of small RNAs derived from Illumina sequencing runs of six *Aedes albopictus* samples. Size distribution and relative frequency in each sample are shown for the small RNAs derived from embryos, larvae, pupae, adult males, sugar-fed adult females, and blood-fed adult females.

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Table 1. Novel miRNAs discovered in *Aedes albopictus*.

Name ¹	Sequence (5'-3')	Contig number ²	Start ³	End ⁴	Strand ⁵
Mosquito-specific					
aal-miR-new1	AAAGCAACCGAACCAATTGTCCA	25767	26526	26448	-
aal-miR-new1*	TGGCTTTTGCTTGGTAGCCTCA	25767	26526	26448	-
aal-miR-new2	ATTAGAATGTGGAATCTGTTTT	21646	21603	21512	-
aal-miR-new2*	AAAAGTTTTTCATATTCTTGCG(G) ⁶	21646	21603	21512	-
Culicinae-specific					
aal-miR-new3-1	TTCCTGACTTATACGCTTACCT	8458	2362	2439	+
aal-miR-new3-1*	TAAGTAGATAAATCAGAAAGA	8458	2362	2439	+
aal-miR-new3-2	TTCCTGACTTATACGCTTACCT	8459	21019	21096	+
aal-miR-new3-2*	TAAGTAGATAAATCAGAAAGA	8459	21019	21096	+
aal-miR-new4	TAAGCAATTTATCGTCTTGAT(G) ⁶	4828	59852	59768	-
Aedes-specific					
aal-miR-new5	AACGTGATGTATGTGAGAAGAG	3687	11751	11663	-
aal-miR-new6	TGTAGAAATGTTCCGGATTTCCGGCT(GAA) ⁶	15327	62795	62885	+
aal-miR-new7	CAGGATTCGAAGTAGGTCAT(GCTA) ⁶	21414	4023	3929	-
aal-miR-new8	GAGGACTAAGCGCATTTTTT	924	44864	44777	-
aal-miR-new9	TCCACTATTAGCCCGAATTGTA	2411	9437	9370	-
aal-miR-new10	GAAGACACCGGAACGATTTGA	24094	20128	20040	-
aal-miR-new11	TTTGACAGTTCTGAAGATGAC	1075	3388	3473	+
aal-miR-new12	ATGGTACATTGAAGTAGGTGAG	22140	9042	8966	-
aal-miR-new13	CTTCATGATGACAACCTTACACA	11123	34944	34853	-
aal-miR-new13*	TATGTTTGTTTCATACTGAT	11123	34944	34853	-
aal-miR-new14	AGGGAAGGCAGTTTGAACAGCGGG(A) ⁶	25387	2495	2423	-
aal-miR-new15	AACCTTAGAAGCTTCAAGGTA	5127	29042	29129	+
aal-miR-new15*	GCCTTGACTGTTTCCTGTT	5127	29042	29129	+

¹The nomenclature of the novel miRNAs is provisional pending further characterization. "-1", and "-2" suffixes refer to different pre-miRNA secondary structures that produce the same mature miRNA. "Mosquito-specific" are those miRNAs found to date only in mosquitoes. "Culicinae-specific" are those miRNAs found to date only in *Aedes* and *Culex* species. "Aedes-specific" are those miRNAs found to date only in *Ae. albopictus* and *Ae. aegypti*.

²Contig number in *Aedes aegypti* assembly version AaegL1.

³location in contig of first nucleotide.

⁴location in contig of last nucleotide.

⁵5'-3' orientation in contig (+, positive strand; -, negative strand).

⁶Shown are the most abundant variants and the longest variants in parentheses.

⁷"*" means "star miRNA".

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preparing samples for Illumina sequencing. Larvae were collected at each instar and pooled to generate early (I and II instars) and late (III and IV instars) larval samples. Female adults were fed on mouse blood and sugar water, and blood-fed samples were collected at 24hrs post-blood-meal. Samples were either homogenized immediately for RNA isolation or flash frozen in liquid nitrogen immediately following collection, then stored at -80°C. Total RNA isolation was carried out using a mirVana miRNA isolation kit (Ambion, Austin, TX). Northern blots were carried out based on Mead *et al.* [12]. Briefly, total RNA was loaded onto 15% denaturing polyacrylamide gels, and run with ssDNA markers 19 and 23 nt in length. The RNA gels were transferred to BrightStar-Plus nylon membranes (Ambion), cross linked with UV, prehybridized, and then hybridized overnight in the ULTRAhyb-Oligo Hybridization Buffer (Ambion) with the appropriate DIG-labeled probe at 42°C. Wash conditions were the same as described in Mead *et al.* [12]. Antisense 5' digoxigenin-labeled miRCURY LNA probes were purchased from Exiqon (Vedbaek, Denmark). Probe sequences were complementary to those shown in Li *et al.* [13].

Results and Discussion

Small RNA Sequencing and Evidence for Transcription of Known miRNAs

Small RNAs were sequenced from a number of developmental stages to increase the likelihood of discovering the full complement of *Ae. albopictus* miRNAs. Small RNA libraries were constructed from embryos, larvae, pupae, adult males, sugar-fed adult females and blood-fed adult females pooled at various time points after feeding. Between 10–14 million high-quality small RNA reads were obtained from each sample after Illumina sequencing and filtering out linker sequences and ambiguous reads (Figure 1). More than 50% of the reads for all six samples were ~22 nt in length as expected for insect miRNAs. There also is an elevated population of small RNAs ~28 nt in length in embryos, larvae and females, which may represent *piwi-interacting* RNAs (piRNAs). The comparisons of these ~28 nt RNAs to the *Ae. albopictus* genome sequence, when available, will help the confirmation of their piRNA status and the identification of their sources. piRNAs are known to derive from and suppress repetitive sequences

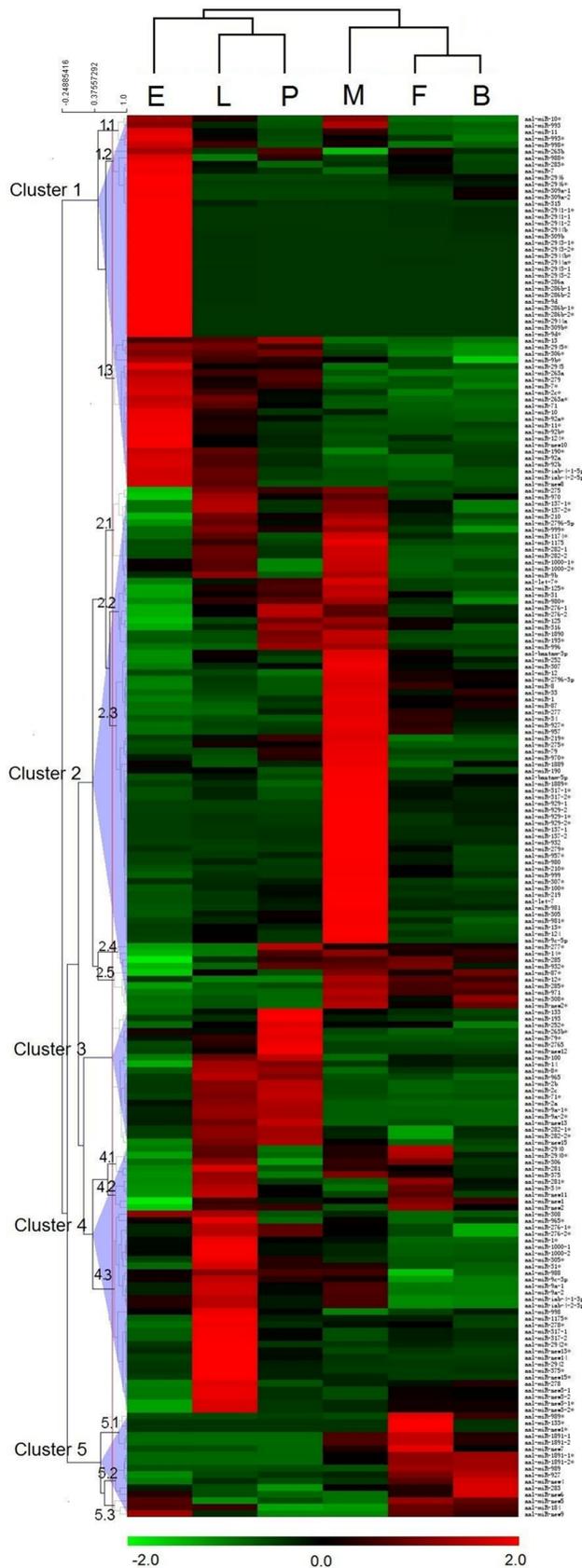


Figure 4. Hierarchical clustering of miRNA expression. Normalized expression profiles of 215 miRNA/miRNA*s from six different

developmental stages were clustered. Stages are in columns and miRNAs in rows. Red indicates that a gene is represented highly at the stage, whereas green indicates the opposite. miRNAs with similar expression patterns cluster together. There are five Clusters (1–5) with variable numbers of sub-clusters. Abbreviations: **E**, embryos; **L**, larvae; **P**, pupae; **M**, adult males; **F**, adult females and **B**, blood-fed adult females.

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including transposable elements [24]. Recently piRNAs have been shown to be involved in suppression of virus infection in mosquitoes [25,26]. Further investigation of the piRNA pathway in *Ae. albopictus* will improve our understanding of how this important vector species may defend against repetitive sequences and viruses.

A total of 104 miRNA genes with sequence similarity to previously-described miRNAs (miRBase v17) were identified in *Ae. albopictus* (Tables S1). All but one of the 88 known *Ae. aegypti* pre-miRNA genes were recovered (Table S1; Figure 2; Li *et al.* [13]; miRBase version 17). *ae-miR-1174*, the only *Ae. aegypti* miRNA that was not represented in any of the *Ae. albopictus* libraries, also is undetectable by northern blot analysis of samples from all developmental stages [27]. However, small RNAs matching *ae-miR-1174** are found, and the putative *Ae. albopictus* orthologue, *aal-miR-1174**, shares 21 nt identity with the mature *ecu-miR-1174* from *Cu. quinquefasciatus*. Thus, members of the miR-1174 family may have undergone an arm switch [28] with respect to its mature and star sequences in different mosquito species. All orthologues miRNAs that could be recovered according to published data were found in this study, supporting the conclusion that the approach used here is comprehensive.

Novel miRNAs

A total of 15 novel miRNAs were found in *Ae. albopictus* by matching small RNA reads with the *Ae. aegypti* genome sequence and performing subsequent bioinformatic confirmation (Table 1; Figure 3). The criteria set forth by Berezikov *et al.* [11] were used to ensure the authenticity of the novel miRNAs. The reliance on matching *Ae. aegypti* pre-miRNAs to confirm the authenticity of the *Ae. albopictus* miRNAs results in finding only those that are conserved between the two species. *Aedes aegypti* and *Ae. albopictus* are relatively closely-related, both belonging to the same subgenus *Stegomyia* [29], thus it is likely that the number of *albopictus*- or *aegypti*-specific miRNAs will be low. Indeed, all of the 88 reported *Ae. aegypti* miRNA genes (miRBase v.17) were found in *Ae. albopictus*. Small RNAs that do not map to the *Ae. aegypti* genome were not investigated because genomic DNA sequencing flanking them are needed to evaluate the folding of the pre-miRNA hairpins. The presence of a pre-miRNA hairpin is a critical requirement for authentic miRNAs [11]. Such unmapped miRNAs, if present, are likely *albopictus*-specific and will be uncovered when the *Ae. albopictus* genome assembly becomes available.

Evolution and Expansion of Mosquito-specific miRNAs

While all mature miRNAs share sequence identity between the two *Aedes* species, there are a few cases of variation in the miRNA* sequences (Figures 2 and 3; Table S1). The *Ae. albopictus* miRNAs that matched known *Ae. aegypti* pre-miRNAs showed identical mature miRNA sequences although there are truncations at the ends as observed in previous reports [12,13]. Three examples, *miR-375**, *miR92b** and *miR-2946**, show one nucleotide difference between *Ae. albopictus* and *Ae. aegypti*, (Table S1, Figures 2 and 3). The majority of the miRNA genes that have homologues in non-mosquito species (Table S1) are conserved in all four mosquito

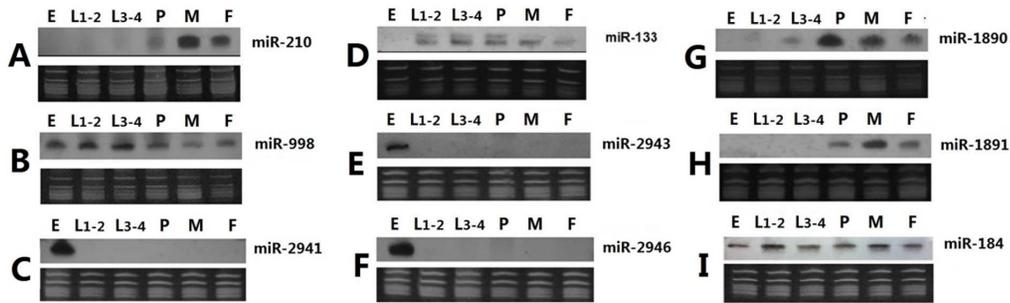


Figure 5. Northern blot analyses of representative miRNAs in *Aedes albopictus*. Nine representative miRNAs, *miR-210* (A), *miR-998* (B), *miR-2941* (C), *miR-133* (D), *miR-2943* (E), *miR-2946* (F), *miR-1890* (G), *miR-1891* (H) and *miR-184* (I) were subjected to northern blot analyses. The top panels are the northern results and the bottom panels are RNA gels for verification of small ribosomal and tRNA integrity and equal loading of total RNA. ssDNA size markers (19 and 23 nts, not shown) also were visualized on the RNA gel for size estimation. Fifteen micrograms of total RNA were loaded for each sample. Abbreviations: **E**, embryos; **L1–2**, mixed 1st and 2nd instar larvae; **L3–4**, mixed 3rd and 4th instar larvae; **P**, pupae; **M**, adult males and **F**, adult females.

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species analyzed, *Ae. albopictus*, *Ae. aegypti*, *Cu. quinquefasciatus* and *An. gambiae*. The two exceptions are *miR-282* and *miR-927*, which are found in *Ae. aegypti* and *An. gambiae* but not in *Cu. quinquefasciatus*, indicating a loss or rapid change of these two miRNAs in the latter species (Table S3). Among the previously-known mosquito-specific miRNAs, *miR-2941* is found only in *Aedes* while *miR-1889*, *miR-2940*, and *miR-2946* are conserved in the Culicinae subfamily, which includes *Aedes* and *Culex* species (Table S1). The rest of the known mosquito miRNAs are conserved in all four mosquito species (Table S3). In contrast, only two of the 15 novel miRNAs are conserved in all four species in the three mosquito genera (Table 1, Table S1). Two additional miRNA genes are conserved in the Culicinae subfamily, and 11 are found only in *Aedes*. Furthermore, all 15 novel miRNAs appear to be unique to mosquitoes and have no sequence similarity to any known miRNAs in the miRBase (v.17) and in the sequenced genomes of *Drosophila* and other insects (data not shown). This may reflect the fact that the accumulation of entries from an ever-increasing number of organisms will exhaust the list of conserved miRNAs and newly-discovered miRNAs are likely to be lineage-specific. The current study increased the number of putative mosquito-specific miRNAs from 10 to 25 (Table S3).

Two observations support the hypothesis that the conservation and lineage-specificity of miRNAs may contain useful information to infer mosquito species phylogeny as was shown recently in other taxonomic groups [30,31]. First, only two of the broadly-conserved miRNAs (*miR-282* and *miR-927*) were either lost or mutated significantly in *C. quinquefasciatus*. This low rate of miRNA loss makes them good phylogenetic markers at the sub-family and genus levels. Second, the pattern of lineage-specific miRNAs is consistent with their phylogenetic relationship. For example, there are 14 *Aedes*-specific (11 novel plus 3 previously known, Table S3) and three Culicinae-specific (*miR-2941*, *miR-new3* and *miR-new4*) miRNAs. However, there are no mosquito-specific miRNAs that are shared by *Aedes* and *Anopheles* alone or *Culex* and *Anopheles* alone. Given the significant involvement of miRNAs in development, these analyses have the potential to link miRNAs to novel evolutionary and developmental features associated with a particular mosquito taxon.

Stage-specific miRNA Expression Profiles

The abundance of transcription from all 119 miRNA genes was evaluated for both miRNAs and miRNA*s on the basis of normalized read counts per miRNA (Table S2; Figure 4). Five

clusters (1–5) and additional sub-clusters were identified. Hierarchical clustering showed that many miRNAs were embryo-specific (sub-cluster 1.2) including three mosquito-specific miRNAs, *miR-2941*, *miR-2943*, and *miR-2946*. The high level of miRNA accumulation in embryos is consistent with that found in *Ae. aegypti* [13], supporting the hypothesis that they may be critical for early development. miRNAs in cluster 4 are larval-specific while miRNAs in cluster 3 are either pupal-specific or expressed highly in both larval and pupal stages. A total of 71 miRNAs and miRNA*s are found predominantly in adult males (cluster 2) while 13 miRNAs and miRNA*s are abundant only in adult females (sub-cluster 5.1 and 5.2). The blood-fed female sample comprises individuals harvested at various time points after the blood meal and therefore it is likely that sensitivity in detecting dynamic changes of miRNA levels is lost. However, three small clusters of miRNAs within clusters 4 and 5 are expressed highly in sugar-fed females but at reduced levels in blood-fed females. Some miRNAs, including the novel *miRNA-new6*, showed a higher expression in blood-fed females than in sugar-fed females.

The relative levels of six phylogenetically-conserved (*let-7*, *miR-133*, *miR-184*, *miR-210*, *miR-9a*, and *miR-998*) and six mosquito-specific (*miR-1890*, *miR-1891*, *miR-1175*, *miR-2941*, *miR-2943* and *miR-946*) miRNAs were confirmed by northern blot analyses (Figure 5). All miRNAs tested showed accumulation in at least one of the five developmental samples. The patterns for all except *miR-9a* are consistent with the Illumina results and it is not clear at this time what caused the discrepancy with *miR-9a*. The Illumina sequencing results also are consistent with *Ae. albopictus* northern blot data reported in Zheng *et al.* [27]. For example, similar to what was shown in Figure 4, the northern blot of *let-7* indicated that it accumulated mainly in developmental stages after pupation (Figure 5; Zheng *et al.* [27]). *let-7* also was shown to accumulate abundantly in the later developmental stages in *D. melanogaster*, *An. stephensi* and *Ae. aegypti* [12,13,32]. Furthermore, *let-7* plays a critical developmental regulatory role in the worm, *Caenorhabditis elegans*, by promoting the development of the 4th instar larva to adult [33].

miRNAs and Mosquito Biology

Demonstrating the functions of the 25 “mosquito-specific” miRNAs is expected to inform studies of mosquito biology and mosquito-specific adaptations. Target predictions have been used as clues to miRNA functions and such predictions for known miRNAs are available already at (www.mirbase.org). Analyses of

all *Ae. albopictus* miRNAs including the 15 novel miRNAs were performed using Miranda [23] and the annotated transcripts from *Ae. aegypti* (Vectorbase). Each miRNA on average has >100 predicted targets at a stringent score of 150 (only the targets of the novel miRNAs are shown in Table S4). Gene Ontology terms associated with these targets were retrieved and enrichment analyses were performed separately for the targets of miRNAs of each of the five major expression clusters (Figure 4). The GO terms of all *Ae. aegypti* transcripts that have annotated 3'-end untranslated regions (UTR) were used as the reference and the results are shown in Figures S1 and S2. These analyses provide the basis for further functional studies to confirm the miRNA-target relationships and to investigate the functions of these miRNAs. For example, targets of miRNAs that are expressed predominantly during the embryonic stage showed significant GO term enrichment in transcription regulation, signal transduction, and cytoskeletal protein binding. The enrichment in these types of genes is consistent with expected molecular processes during embryonic development. The UTR and GO annotation is currently incomplete for the *Ae. aegypti* genome. Potential mRNA targets will not be identified unless their 3'UTRs are annotated. Nonetheless, GO terms of 6741 transcripts are available for analysis. Therefore the incompleteness of UTR and GO annotation should not significantly affect the general conclusion of the GO analysis described above, unless there is a systemic bias in the annotation of a certain class of genes.

Female mosquitoes feed on blood to acquire proteins necessary for reproduction and nutrition. Transmission of vector-borne pathogens requires blood feeding on an infected host and the blood meal also triggers a cascade of endocrinological, molecular, and physiological events that switch the adult female from host-seeking to reproduction [34,35]. Therefore it is not unexpected to detect increases or decreases in accumulation of the majority of 119 miRNAs post blood-feeding. These miRNAs are likely important regulators of blood-meal-induced molecular changes. For example, *aae-miR-275* affects blood digestion, fluid secretion, and egg development in *Ae. Aegypti* [36]. *Aedes albopictus* has undergone a rapid expansion in its world-wide distribution and it is emerging as a vector for Dengue and Chikungunya viruses [14]. Understanding the function of a key class of gene regulators in this important mosquito vector has biological and practical significance.

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Supporting Information

Figure S1 Color-coded gene ontology (GO) graph showing significantly-enriched GO terms describing biological processes. Predicted targets of miRNAs within each cluster were analyzed separately. A false discovery rate (FDR) of 0.01 was used as the threshold. For each GO term, a brief description, GO number, FDR and P value were shown.

(PDF)

Figure S2 Color-coded gene ontology (GO) graph showing significantly enriched GO terms describing molecular functions. Predicted targets of miRNAs within each cluster (Figure 4) were analyzed separately. A false discovery rate (FDR) of 0.01 was used as the threshold. For each GO term, a brief description, GO number, FDR and P value were shown. No GO term was enriched significantly for targets of miRNAs in cluster 3 at a FDR of 0.01.

(PDF)

Table S1 Sequence, location, expression, and hairpins of miRNAs in *Aedes albopictus*.

(XLSX)

Table S2 Number of small RNA reads mapped to a miRNA/miRNA*.

(XLS)

Table S3 Distribution of mosquito-specific miRNAs as indicated by Mapmi values.

(XLSX)

Table S4 Predicted targets of the 15 novel miRNAs. Note that targets for miRNA* that showed >15 total hits in the 6 samples are also shown.

(XLS)

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Author Contributions

Conceived and designed the experiments: XC ZT. Performed the experiments: JG WH JW PZ. Analyzed the data: MC JG WH JW AAJ XC ZT. Contributed reagents/materials/analysis tools: MC. Wrote the paper: JG WH JW AAJ XC ZT.

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