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Article

The microRNAs in the Antennae of *Apolygus lucorum* (Hemiptera: Miridae): Expression Properties and Targets Prediction

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Abstract: MicroRNAs (miRNAs) are a class of small non-coding RNAs, which function as regulators of gene expression and contribute in numerous physiological processes. However, little is known referring to miRNAs function in insect chemosensation. In the current study, nine small RNA libraries were constructed and sequenced from the antennae of nymphs, adult males and females of *Apolygus lucorum*. In total, 399 miRNAs were identified including 275 known and 124 novel miRNAs. Known miRNAs were classified into 71 families, amongst which, 23 families were insect-specific. Expression profile analysis showed that miR-7-5p_1 was the most abundant miRNAs in the antennae of *A. lucorum*. Altogether, 69708 target genes related to biogenesis, membrane and binding activities were predicted for 399 miRNAs. Particularly, 15 miRNAs were found to target 16 olfactory genes. These miRNAs could be involved in regulation of olfactory-associated genes expression. Comparing the antennae of nymphs, adult males and females, 94 miRNAs were found to be differentially expressed. The expression levels of some differentially expressed miRNAs measured by qPCR were consistent with sequencing results. This study provides a global miRNAs transcriptome in the antennae of *A. lucorum* and valuable information for further investigation on miRNA-mRNA interactions, especially the functions of miRNAs in regulating chemosensation.

Keywords: Small RNA sequencing, miRNAs, target prediction, chemosensory-associated genes, *Apolygus lucorum*

1. Introduction

Gene expression is generally regulated by transcriptional and post-transcriptional mechanisms. MicroRNAs (miRNAs), the key regulators in the post-transcriptional complex, are endogenous, ubiquitous, non-coding and small single-stranded RNAs with the length of approximately 18–30 (average size, 22) nucleotides (nt) [1-4]. miRNAs are encoded in the genome and are transcribed by RNA polymerase II in the nucleus as primary RNAs (pri-miRNA). Pri-miRNAs are processed in the nucleus by Drosha/DGCR8 complex to form approximately 70 nt hairpin precursor miRNAs (pre-miRNAs). These transcripts are then transferred into the cytoplasm by Exportin5 and processed by Dicer to yield double-stranded small RNAs. Mature miRNAs are single-strand RNA which load onto the RNA-induced silencing complex (RISC) and mediate target gene transcripts. Expression levels of miRNA transcripts alter by both gene polymorphisms and environmental elements [4-6].

miRNAs are evolutionarily conserved in diverse eukaryotic lineages, and play major roles in various physiological processes including development, reproduction, behaviors, apoptosis, cell differentiation and so on. miRNA function as post-transcriptional and translational regulator by binding to the untranslated region (UTR) or coding sequence (CDS) of messenger RNA (mRNA) of target gene [7-11]. miRNA-mRNA interaction

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interferes with translational performance and initiates the recruitment of mRNA decay factors, preventing or altering the protein expression [4, 7, 12]. Translational suppression or mRNA degradation occurs via perfect or imperfect complementary base-pairing of target seed match sites (2-8 nucleotides) to sequences within UTR (5′ or 3′) or coding regions [5, 13, 14].

The first miRNA, lin-4, was discovered in the larval stage of nematode, *Caenorhabditis elegans*, in 1993. The second miRNA, let-7a, was identified seven years later (2000) in the same nematode and found to have homologous sequences in human and most of the living species [5, 15]. With the development of experimental technologies such as high-throughput RNA sequencing and the progress of computational biology, thousands of miRNAs have been found from plants, animals and various microorganisms and their structures, biosynthesis and functional mechanisms have been characterized [5, 16]. In insects, first miRNAs were reported from the fruit fly, *Drosophila melanogaster* in 2001 [17], however, investigations on the identification and functional characteristics of model and non-model insect miRNAs are limited to recent years. Insect miRNAs contribute in numerous biological functions including metamorphosis and molting, sex determination, reproduction (ovarian development and oogenesis), immunity, wing polyphenism and development, pesticide resistance, chemical communication (olfaction and gustation) and so on. [18-33]. In *D. melanogaster*, miR-8 regulates the transcript of gene associated with the biosynthesis of juvenile hormone (JH) and alters the cell growth in the corpus allatum (CA), causing pupal mortality [34]. The miR-184, targeting the highly conserved CYP303A1, mediates the molting process in *Locusta migratoria*. Additionally, overexpression of miR-184 leads to abnormal molting, while down-regulation causes molting deficiency [35]. Resistance to nitenpyram has been recently reported in *Nilaparvata lugens*. Two miRNAs (novel-mir85 and novel-mir19) down-regulate the transcript levels of the CYP6ER1 and, have been proved to be involved in the resistance pathway to nitenpyram [29, 36].

The green mirid bug, *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae) is one of the most destructive agricultural pests which is known for having a diverse range of hosts (more than 200 plant species) and being widely distributed across the world [37]. In China, mirid bugs were historically secondary pests but turned to be more problematical since the adaptation of *Bt* cotton (1997) and reduction of pesticides usage and therefore considered as serious primary pests [38-42]. The effective suppression of *A. lucorum* and other bugs population in cotton and other crops has been remained challenging mainly due to the high-mobility and host-plants transferring behaviors which occurs locally and seasonally among various crops. Studies have shown that these behaviors predominantly depend on interactions between the host plant volatiles and their highly-sensitive olfactory system [43-46]. However, a comprehensive and practical prospective deciphering this complex interaction in molecular level is still lacking. The host-plant volatiles interact with the olfactory-associated proteins which are mostly expressed in the antennae, the main perceptive peripheral olfactory organs. The odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), ionotropic receptors (IRs), sensory neuron membrane proteins (SNMPs) and gustatory receptors (GRs) are the main chemosensory proteins [47, 48]. Recently, large sets of chemosensory genes including OBPs, CSPs, ORs, IRs, GRs and SNMPs have been reported in the antennae and legs of *A. lucorum* [49-55].

In compare with multiple biological functions, there are few reports referring to the roles of miRNAs in insect chemical communication and the identification and functional analysis of miRNAs in insect chemosensory organs are limited to few species [31-33, 56-60]. In *Drosophila*, CO_2 detecting neurons are naturally exist in the antennae and lack in the maxillary palps (MPs). In the miR-279^{-} mutant population, the transcription factor Nerfin-1 was expressed in the high level and CO_2 neurons were formed in MPs. The miR-279 plays a critical function in the localization of CO_2 neurons and receptors and consequently, CO2-mediating behavior [32]. Furthermore, miR-276a expressed in mushroom body and ellipsoid body mediates the memory formation and responses to odor, respectively, by regulating the transcript of Dopamine receptor (DopR) [56]. In *L. migratoria*,

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DopR1−miR-9a−adenylyl cyclase 2 complex shape the olfactory behavior of locust regarding aggregation. miR-9a up-regulation and down-regulation in gregarious and solitarious individuals, respectively, reduces and increases the behavioral tendency towards aggregation compounds [57]. In our previous work, we described the miRNAs characteristics in the antennae of parasitoid wasp *Microplitis mediator* by identifying the known and novel miRNAs, and determining the expression and targets prediction [58].

In the current study, miRNAs properties in the antennae of *A. lucorum* were investigated. Nine small RNA libraries were prepared from the antennae of nymph, male and female individuals. High-throughput small RNA sequencing and bioinformatics analysis were employed to determine the known and novel miRNAs. The transcript levels of identified miRNAs were evaluated and potential target mRNAs were predicted. In particular, putative miRNAs targeting olfactory-associated genes were identified. Moreover, differentially expressed miRNAs among antennal libraries of nymph, adult male and adult female and their target genes were explored and verified by real-time quantitative polymerase chain reaction (qPCR). The outcomes of the current study deliver a profound understanding of the putative functional role of miRNA in the antennae of *A. lucorum* and create a database for future researches.

2. Results and Discussion

2..1. The summary of small RNA-sequencing data analysis

In order to fully characterize the miRNAs in the antennae of *A. lucorum*, nine small RNA libraries were constructed and sequenced from the nymph, adult male and adult female individuals in triplicate. After processing the raw reads, an average of 26156882, 27126902 and 27633525 clean reads were obtained from nymph, adult male and adult female antennae, respectively. The Q20 percentages of clean reads were ~ 90% (Table S1). For nymph, adult male and adult female 91.63, 86.08 and 84.69 % of all clean reads were mapped to the *A. lucorum* genome, respectively (Table S2). In all libraries, the clean tags were shown to have length distribution of 18-30 bp with two distinct picks, which are consistent with other reports (Figure 1A). The largest groups were accounted for 21-24 nt, implying that the major groups of small RNAs in each library were miRNAs. The second groups were accounted for 27-29 nt, suggesting the abundance of PIWI-Interacting RNAs (piRNAs) in each library. piRNAs are typical type of small RNAs, which function as epigenetic and post-transcriptional silencer of transposable elements in germ line cells [61]. Moreover, the proportional analysis demonstrated that 14.1, 21.1 and 17.4% of total clean reads in nymph, adult male and adult female, respectively were classified as known miRNAs (Figure S1). Based on the UMI-counts, pairwise comparisons of all identified sRNAs between any two libraries demonstrated Pearson correlation values of higher than 0.78, indicating the reliable integrity in transcript measurement among all constructed libraries and biological replicates (Figure S2).

2.2. Identification of known and novel miRNAs

In order to characterize the known miRNAs in the antennae of *A. lucorum*, the clean tags from nine libraries were mapped against Hexapoda miRNAs (miRbase, Release 22.1). In total, 275 unique known miRNAs were identified from the antennae of nymphs, adult males and adult females, amongst which, 153 miRNAs were found to be shared among all libraries. The list of all identified known miRNAs are given in the Supplementary file 1. Eight known miRNAs (miR-252, miR-3024, miR-306_2, miR-316-5p_1, miR-317-3p_1, miR-5735-3p, miR-9b-5p, miR-9e-3p) were only found in adult female libraries and 9 known-miRNAs (miR-13a-3p, miR-2788-3p, miR-278_3, miR-281-3p_7, miR-3041, miR-3049-5p_1, miR-3049-5p_1, miR-6012-3p, miR-7a) were only identified in adult male libraries. In contrast, miR-252b_1 was only identified in nymph libraries. In addition to the known miRNAs, 124 unique novel miRNAs were predicted from all libraries by miRDeep2 software package, amongst which, 61 novel miRNAs were common in all nine libraries. The list of all identified novel miRNAs are given in Supplementary file 1. Nine novel miRNAs (novel_mir38, novel_mir64, novel_mir70, novel_mir73, novel_mir79,

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novel_mir82, novel_mir100, novel_mir104, novel_mir121) were only detected in adult female libraries and 6 novel miRNAs (novel_mir67, novel_mir80, novel_mir83, novel_mir84, novel_mir96, novel_mir112) were only found in adult male libraries. In compare, 6 novel miRNAs (novel_mir36, novel_mir49, novel_mir51, novel_mir91, novel_mir114, novel_mir122) were only identified in nymph libraries.

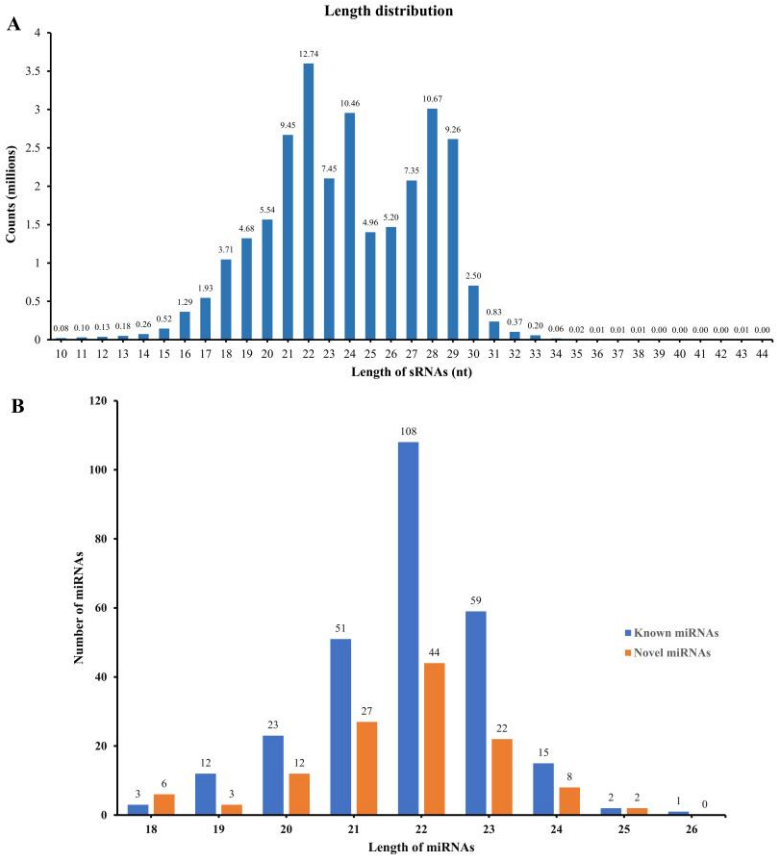

Figure 1. **(A)** Length distribution of small RNA (sRNAs) reads in the antennae of *Apolygus lucorum*. The data are based on the average of 9 libraries. The values on the top of the bars demonstrate the percentage of each length. **(B)** Length distribution of known and novel miRNAs in the antennae of *Apolygus lucorum*. The values on top of the bars demonstrate the number of identified miRNAs for each length.

It has been demonstrated that miRNAs presented in insects are developmental stage- and sex-biased. Some miRNAs are unique for a gender or a distinct developmental stage or even a tissue. In agreement with our results, in the antennae of the mosquito *Aedes aegypti*, miR-34, miR-79, miR-929, and miR-965 were uniquely expressed in the females, whereas miR-92b was especially found in males [60]. Generally, female and male specific miRNAs may be associated with sexual differentiations or gender-biased functions. On the other hands, miRNAs specifically presented in immature stages of hemimetabolous and holometabolous insects could be involved in the developmental regulations and transition [13, 22, 62, 63].

In compare with previously published works related to identification of miRNAs in the insect antennae, we have identified a larger repertoire of miRNAs (399 miRNAs). There are 99 miRNAs in the antennae of the beetle *Holotrichia parallela* (Scarabaeidae) [59]. Previously, we identified 342 miRNAs in the antennae of *M. mediator* [58]. Identification of miRNAs could be influenced by several factors such as availability of genome sequence, genome size and structure, sample collection, small RNA sequencing quality, computational processing and miRNA annotation. The number of identified miRNAs even within the same organism was shown to be different in some cases [9, 64, 65].

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The length distribution of the identified known and novel miRNAs was ranged from 18–26 and 18–25, respectively, showing the peak at 21–23 nt (Figure 1B), which is the most common properties of animal miRNAs [4]. These results demonstrate the creditability of identified known and novel miRNAs in our experiments and consistency with other insect miRNAs [13, 66]. The base distribution analysis of the first and each position of miRNAs reads demonstrated that uridine (U) followed by guanine (G) were more frequently presented than adenine (A) and cytosine (C) (Figure 2). Comparing these results with previous reports [58, 59] it can be suggested that base distribution in insect miRNAs relatively follows the similar trends.

2.3.

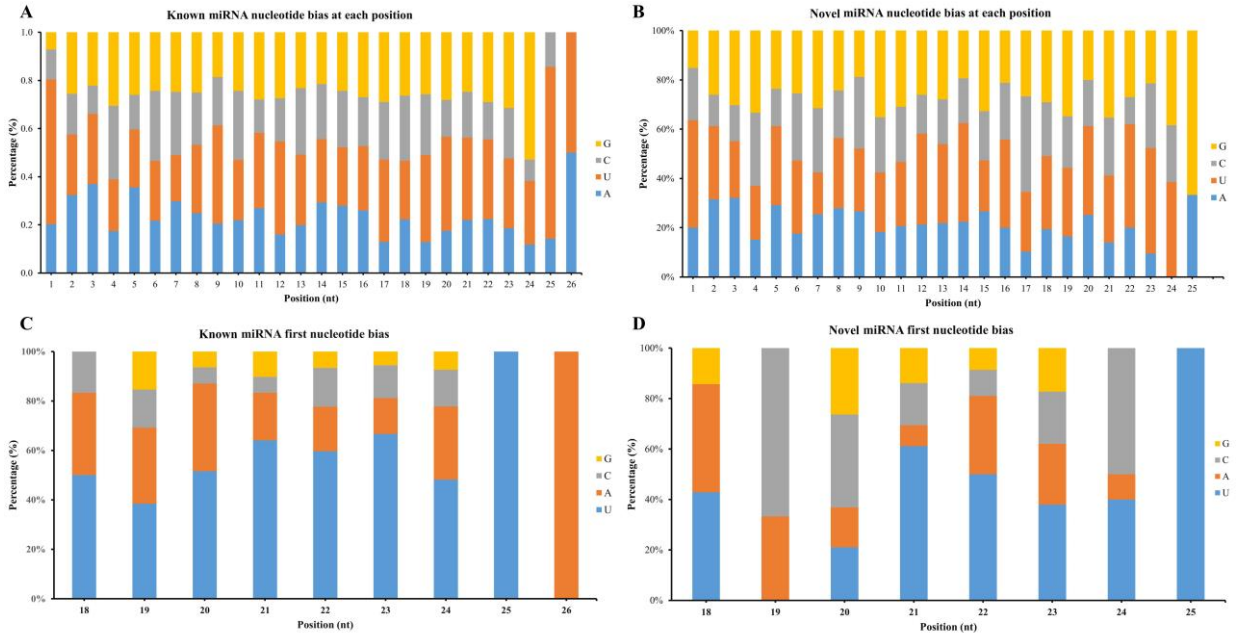

Figure 2. Frequency of the each and first base of identified miRNAs in the antennae of *Apolygus lucorum*. Nucleotide bias of **(A)** known and **(B)** novel miRNAs at each position. First nucleotide bias of **(C)** known and **(D)** novel miRNAs. Y axis demonstrates the percentage of base distributions at each or first position of miRNAs. X axis shows the base position of miRNAs.

Family analysis of known miRNAs

Based on the miRNAs sequence homology comparison with latest release of miRbase [67], family analysis demonstrated that all 275 identified known miRNAs were classified into 71 families, amongst which, six families namely miR-2, miR-276, miR-263, miR-92, miR-281 and miR-9 possessed members more than 10. Whereas, number of miRNAs in other 65 families were ranged from 1–10 members. The miR-2 with 17 members was identified as the largest family in *A. lucorum* antennae (Figure 3). Consistently, previous studies demonstrated that the members of the miR-2, miR-9, miR-92 and miR-263 families were enriched in the insect antennae [58-60]. Interestingly, 48 out of 71 families including the above-mentioned 6 families were found to be conserved across the different phylum of animals. The presence of conserved miRNAs in animals conveys that miRNAs fulfilled significant roles throughout the evolution [68]. On the other hand, 23 families were found to be insect-specific and shown to regulate various functions in insect species.

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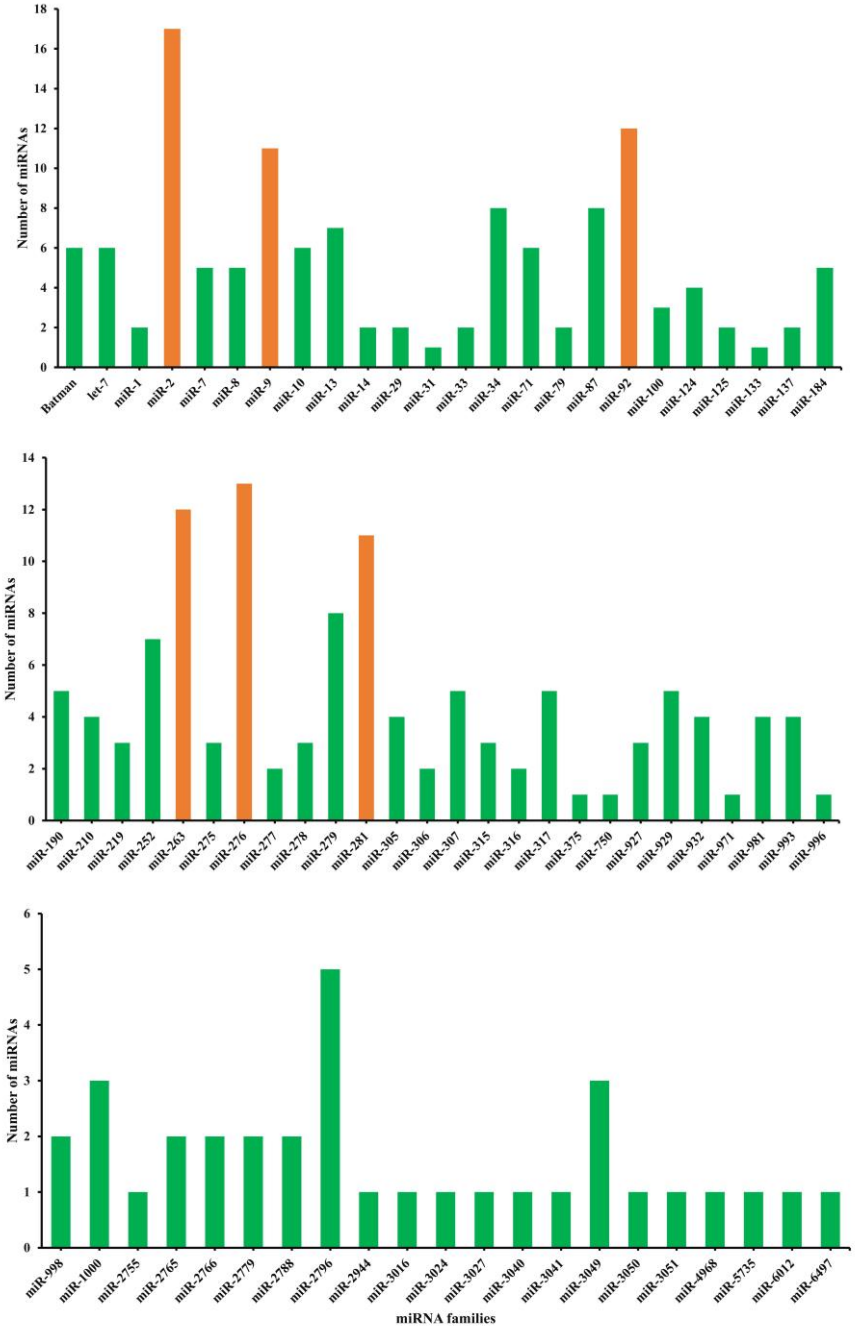

Figure 3. Number of identified known miRNAs in the antennae of *Apolygus lucorum* in different families of miRNAs. The families with members more than ten are shown in orange color.

Of those insect-specific groups, miRNAs from 7 families (miR-3016, miR-3024, miR-3027, miR-3040, miR-3041, miR-3049, miR-3050, miR-3051) are only reported in aphids, and suggested to be Hemiptera-specific miRNAs [69, 70]. The members of miR-2766 family are reported to be specific for lepidopteran insects [71] and our result is the first report of this family in other insect orders.

2.4. Read counts and expression analysis of miRNAs

In each library, the read numbers of known and novel miRNAs were characterized by UMI-count. To increase the reliability of the data, miRNAs with low copy number (UMI-count<10) have been deleted from each library. The expression levels of miRNAs were normalized according to the TPM formula. Based on the average of three biological

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replicates, the top 20 known and novel miRNAs with the highest transcript levels were evaluated for nymph, adult male and adult female libraries. The UMI-counts and the transcript levels of the most abundant known and novel miRNAs were shown to be consistent among the biological replicates. In all nine libraries, miR-7-5p_1 with the count number of ~120000 and the TPM of ~5000 was the most abundant known miRNAs. In contrast, novel_mir1 with the count number of 25416 and 24394 and TPM of 1173 and 1066 was the most abundant novel miRNA in the antennae of adult male and adult female libraries, respectively, while novel_mir2 (count: 58163, TPM: 2604) was the most highly expressed novel miRNA in the antennae of nymph. The top 20 most abundant miRNAs (known and novel) from nymph, adult male and female libraries are separately listed in Table S3-S8.

Several of known miRNAs, highly expressed in the antennae of *A. lucorum* such as miR-7-5p, miR-263a, let-7-5p and miR-8, were also found to be highly expressed in other insect species such as *M. mediator* [58], *Plutella xylostella* [72] and *Apis mellifera* [73]. In nymphs, adult male and female, top 20 known miRNAs with the highest transcript levels accounted for 81.85%, 81.04% and 81.85% of total counts, while for novel miRNAs, 82.58%, 89.94% and 83.41% of total reads were recorded for top 20 highly expressed ones, respectively. Comparingly, the identified known miRNAs were expressed at higher levels than those of novel miRNAs (Table S3-S8, Supplementary file 1), which is consistent with other studies [58, 65].

Of 20 highly expressed known and novel miRNAs in nymph, male and female antennae, 15 miRNAs were shared. The relatively similar expression pattern of the most abundant known and novel miRNAs could be associated with similar functions in different developmental stages and sexes. The regulations of insect miRNAs in different developmental stages have been targeted in several studies. For instance, in the hemipteran insect, *N. lugens*, the miRNAs profiles in the whole body of fifth instar nymph, adult male and adult female indicated that the expressions of miRNAs were widely varied among the developmental phases. Several miRNAs were found ubiquitously in nymph, male and female, while some of them were highly specific in one group. Additionally, there were several ubiquitous miRNAs, which their transcript levels were significantly dissimilar among the developmental stages [74]. These results are in a good agreement with our finding. Here, we have identified some miRNAs which shown to be specific for nymph, male or female antennal libraries. The specificity of those miRNAs needs to be verified by further studies. However, the average read count of biological replicates of all male, female and nymph specific miRNAs were ranged from 10–50. Therefore, it could be implied that some of those miRNAs maybe not detected in other libraries due to low copy number.

2.5. Prediction of chemosensory associated miRNAs

To identify the miRNAs targeting chemosensory-associated genes, prediction was carried out by miRanda and RNAhybrid softwares. In overall, 69,708 targets were predicted for 399 known and novel miRNAs by combination of both softwares. Particularly, 15 unique miRNAs were found to target 16 unique olfactory genes (Table 1). Three olfactory genes (AlucCSP1, AlucCSP9 and AlucOBP2) were targeted by a same miRNA (novel_mir90). In comparison, AlucOR7 was targeted by two miRNAs (miR-34 and miR-6012-3p). Of 17 female-specific miRNAs, novel_mir70 and novel_mir82 were found to target AlucOR86 and AlucIR75d, respectively. Of 15 male-specific miRNAs, miR-6012-3p and novel_mir83 were shown to target AlucOR7 and AlucOR31, respectively. Of 7 nymph-specific miRNAs, novel_mir36 and novel_mir49 were associated with AlucCSP4 and AlucIR75c, respectively. Of top 20 most expressed miRNAs, novel_mir21 targeted AlucCSP11. Target site analysis indicated that miRNAs-olfactory genes interactions mainly occurred in CDS (Table 1).

In the previous study, we predicted a total of 33 miRNAs, targeting olfactory-associated genes in the antennae of *M. mediator*. As an example, MmedOR118 was targeted by miR-6012-3p [58]. In the current work, we predicted that this miRNA targets AlucOR7. In the antennae of *A. aegypti*, miR-34-5p specifically expressed in the female antennae targeted multiple olfactory-associated genes including AeagOR2, AeagOR52, AeagOR81,

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AeagOBP58 and AeagIR107.1. Likewise, miR-92b-3p uniquely expressed in the male antennae targeted several olfactory-associated genes including AeagOR49, AeagOBP11, AeagIR7h.2 and AeagIR41e [60]. In our analysis, AlucOR7 and AlucOBP31 were identified as the putative targets of miR-34 and miR-92b-3p, respectively. Here, we found that the miRNAs of *A. lucorum* also can target the members of major olfactory gene families. Therefore, it could be suggested that the above-mentioned miRNAs and members of their families may have regulatory functions in insect chemosensation, however, none of them being functionally investigated, yet. Only in *D. melanogaster* larvae, knocking-out of miR-375 and miR-1000 significantly minimized the olfactory (appetitive and aversive) memory formation [75].

Table 1. Candidate miRNAs targeting olfactory-associated genes in the antennae of *Apolygus lucorum*

miRNA ID	Target Gene ID	RNAhybrid MEF	RNAhybrid P-value	miRanda MEF	miRanda score	Binding site
miR-100-5p	AlucOBP4	-31.1	0.0084	-30.35	163	3'-UTR
miR-3040	AlucOBP9	-38.2	0.0006	-33.59	175	3'-UTR
miR-34	AlucOR7	-32.2	0.0491	-25.82	161	CDS
miR-6012-3p	AlucOR7	-32	0.0070	-29.82	167	CDS
miR-6497-5p	AlucCSP10	-34.7	0.0172	-31.43	167	CDS
miR-92b-3p_1	AlucOBP31	-29.4	0.0368	-25.2	162	CDS
novel_mir111	AlucCSP11	-29.3	0.0093	-27.5	168	CDS
novel_mir21	AlucOR34	-31.6	0.0416	-31.96	163	CDS
novel_mir36	AlucCSP4	-29.4	0.0354	-25.34	167	CDS
novel_mir49	AlucIR75c	-33	0.0069	-50.13	327	CDS
novel_mir70	AlucOR86	-34.5	0.02961	-26.53	171	CDS
novel_mir82	AlucIR75d	-31.2	0. 0097	-26.88	173	CDS
novel_mir83	AlucOR31	-31	0. 0048	-28.12	171	CDS
novel_mir90	AlucCSP1	-29.6	0.0160	-27.59	156	CDS
novel_mir90	AlucCSP9	-30	0.0171	-28.12	157	CDS
novel_mir90	AlucOBP2	-29.1	0.0054	-26.71	154	5'-UTR
novel_mir95	AlucOR12	-31.7	0.0485	-28.71	170	CDS

2.6. Differentially expressed miRNAs in the antennae of nymph, adult male and female

To characterize differentially expressed miRNAs (DEmiRNAs) in the antennae of *A. lucorum*, the transcripts of miRNAs in different groups were compared by adjusted *P*-value (< 0.05) and Log2 fold change (|≤ 1|). Comparing the miRNA transcript levels in libraries of the nymphs, adult males and adult females, 94 miRNAs (58 known and 36 novel) (23.55% of total identified miRNAs) were shown to be significantly differentially expressed (Figure 4A). By analysis of DEmiRNAs in nymph versus (vs.) adult male group, 74 DEmiRNAs (44 known and 30 novel) were identified, amongst which, 41 miRNAs were up-regulated and 33 miRNAs were down-regulated. The highest log2 fold change (-8.32) was recorded for down-regulated miR-71-5p (Figure 4B, Table S9). In nymph libraries vs.

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adult female libraries 59 DEmiRNAs (39 known and 20 novel) were identified, amongst which, 35 miRNAs were up-regulated and 24 miRNAs were down-regulated. The highest log2 fold change (6.89) was recorded for up-regulated novel_mir90 (Figure 4C, Table S10). Finally, comparison of adult male libraries and adult female libraries revealed that only 6 miRNAs (4 known and 2 novel) were differentially expressed, of which 4 miRNAs were up-regulated and 2 miRNAs were down-regulated. The highest log2 fold change (3.65) was recorded for up-regulated miR-263b-5p_1 (Figure 4D, Table S11).

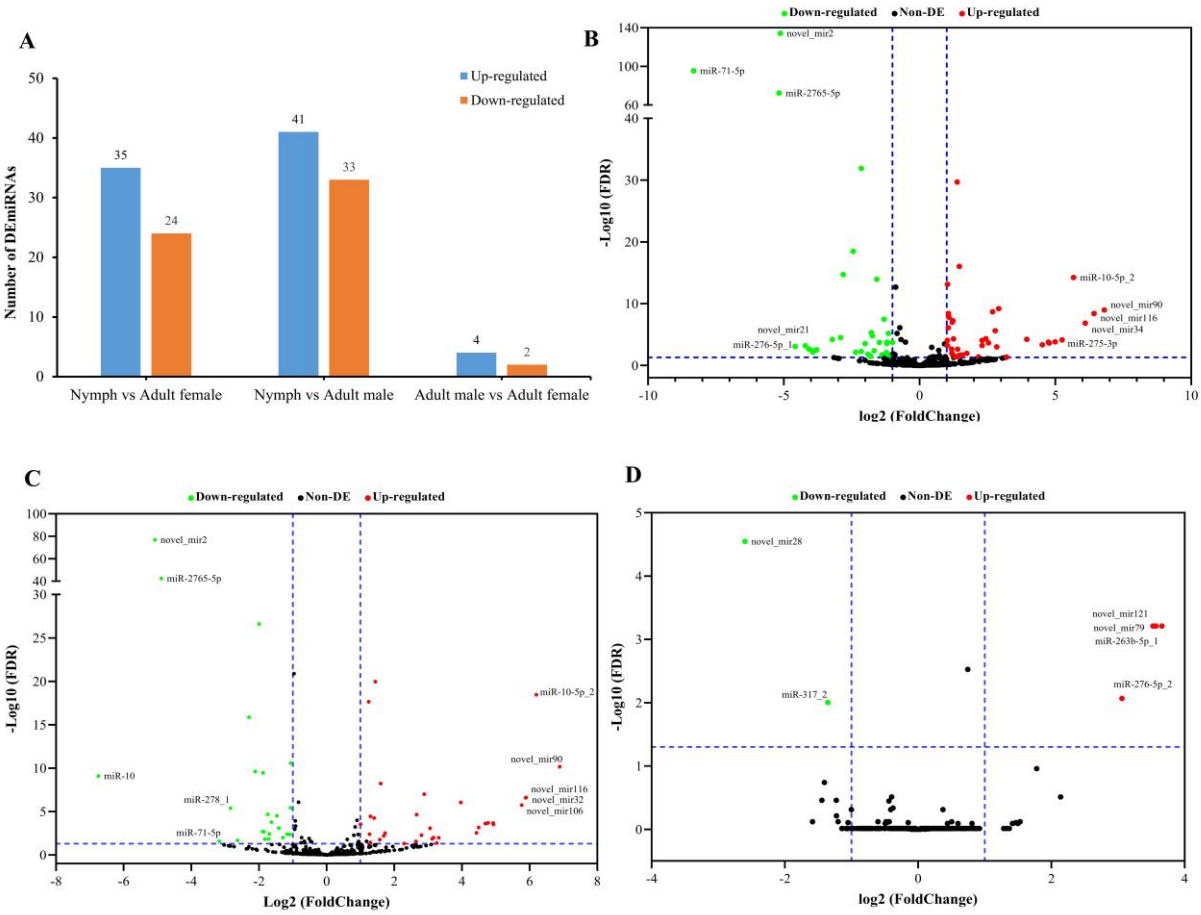

Figure 4. Analysis of differentially expressed miRNAs (DEmiRNAs) in the antennae of *Apolygus lucorum*. **(A)** Numbers of DEmiRNAs identified in different comparisonal groups. **(B)** The volcano plot of DEmiRNAs in libraries of nymph vs. adult female. **(C)** The volcano plot of DEmiRNAs in libraries of nymph vs. adult male. **(D)** The volcano plot of DEmiRNAs in libraries of adult male vs. adult female. Green and red dots demonstrate down-regulation and up-regulation, respectively. Black dots indicate miRNAs with no changes in expression levels.

2.7. Verification of DEmiRNAs

To verify the expression profile of miRNAs, determined by small RNA sequencing, a total of 14 DEmiRNAs from different comparison group were selected for qPCR. The results demonstrated that in nymph vs. adult male comparison group, four out of five selected DEmiRNAs had the same expression patterns as detected in small RNA sequencing analysis, while no significant differences were found in the expression level of novel_mir34 between the antennae of nymph and adult male (Figure 5A, B). In nymph vs. adult female comparison group, all five selected DEmiRNAs showed the same expression pattern as observed in the sequencing data (Figure 5C, D). Finally, comparing the adult male and adult female by qPCR revealed that four out of five DEmiRNAs had similar expression patterns with sequencing data, while only novel_mir121 displayed no significant difference in transcript level between adult male and female antennae (Figure 5E, F).

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In overall, it could be concluded that the expression profiles of selected DEmiRNAs measured by qPCR were relatively consistent with the small RNA sequencing results, suggesting the credibility and repeatability of sequencing data.

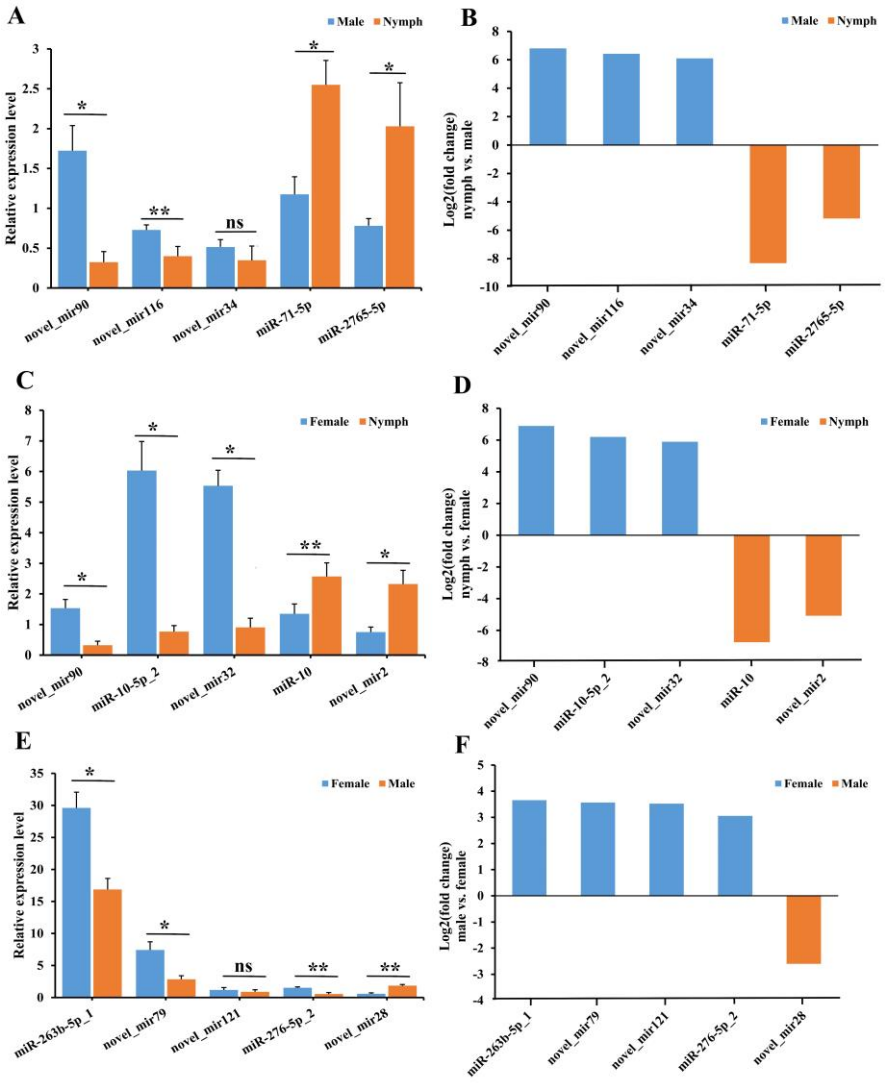

Figure 5. Comparisons of analysis of some differentially expressed miRNAs in the antennae of *Apolygus lucorum* resulted from qPCR measurements and small RNA sequencing data. **(A)** Relative expression levels of DEmiRNAs in the antennae of nymph and adult male measured by qPCR. **(B)** Fold-change (log2) analysis of DEmiRNAs in nymph vs. adult male antennal libraries based on the sequencing data. **(C)** Relative expression levels of DEmiRNAs in the antennae of nymph and adult female measured by qPCR. **(D)** Fold-change (log2) analysis of DEmiRNAs in nymph vs. adult female antennal libraries based on the sequencing data. **(E)** Relative expression levels of DEmiRNAs in the antennae of adult male and adult female measured by qPCR. **(F)** Fold-change (log2) analysis of DEmiRNAs in adult male vs. adult female antennal libraries based on the sequencing data. For A, C and E, results were analyzed using the Student's t-test. "*" and "**" indicate the significant differences at $P < 0.05$ and $P < 0.01$ levels, respectively; "ns" indicates no significant differences. For B, D and F, the blue and the orange columns above and below X-axis demonstrate up-regulation and down-regulation, respectively.

2.8. Targets prediction and functional analysis of DEmiRNAs

To better understand the functional contributions of DEmiRNAs in the antennae of *A. lucorum*, miRanda and RNAhybrid were utilized to predict the candidate target genes for 94 DEmiRNAs. In nymph vs. adult female libraries, 59 DEmiRNAs targeted 11895 genes. In nymph vs. adult male libraries, 74 DEmiRNAs, targeted 14965 genes. Noteworthy, miR-34 targeting AlucOR7 and novel_mir90 targeting AlucCSP1, AlucCSP9 and AlucOBP2 were differentially up-regulated in both groups. In contrast, novel_mir21 targeting AlucOR34 was found to be differentially down-regulated in nymph vs. adult male group.

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Six DEmiRNAs in adult male vs. adult female libraries targeted 480 genes, none of which were chemosensory-associated genes.

GO annotation indicated that 12911, 10952, and 435 GO terms were enriched on the target genes of DEmiRNAs in nymph vs. male, nymph vs. female and male vs. female groups, respectively. In biological process class, the target genes of DEmiRNAs were predominantly related to biogenesis cellular, metabolic and single organism processes. Membrane, cell and cell part related genes were mainly enriched in cellular component category. In molecular function group, the most abundant target genes were annotated for binding, catalytic activity and structural molecule activity. Comparing all groups, larger number of target genes were annotated under the cellular component category. However, the binding related genes were accounted for the highest number of DEmiRNAs targets among Go terms (Figure S3A-S5A).

To further understand the correlation between the DEmiRNAs and their candidate targets, for each comparison group, top 5 up- and down-regulated miRNAs and some of the corresponding target genes were selected to create miRNA-mRNA networks (Figure S6). Furthermore, KEGG annotation was performed to identify top 20 enriched functional pathways of target genes regulated by DEmiRNAs. Based on the Qvalue and RichFactor, in nymph vs. female and nymph vs. male group, Apoptosis-fly and Mitophagy-animal were shown to be the most enriched pathway. In contrast, for male vs. female group, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis was shown to be the most enriched pathway (Figure S3B-S5B).

3. Materials and methods

3.1. Insect rearing and tissue collection

An established laboratory colony of *A. lucorum* were taken from the National Plant Protection Scientific Observation and Experiment Station (Langfang, Hebei, China). The nymphs and adults were reared under controlled conditions (28 ± 2°C, 65 ± 5% R.H., 14L / 10D) on clean and new-harvested corn eras and green bean pods, respectively. The food sources were replaced with fresh ones every other day and a 10% honey solution was provided as supplementary nutrition. Last instar nymphs (1–3 days before eclosion) and newly emerged male and female adults (1–3 days after eclosion) were selected for tissue collection. Insects were first anesthetized by CO_2 and the antennal samples (150 pairs for adults and 200 pairs for nymph) were dissected, transferred to liquid nitrogen, and stored at –80 °C for subsequent usage. Tissue samples were prepared in three replicates.

3.2. RNA isolation and library preparation and sequencing

Trizol reagent (Invitrogen, CA) was used to isolate the total RNA from the antennae of nymph, male and female individuals. RNA samples were then treated with DNaseI (NEB, USA) for 30 min at 37°C and quantified by NanoDrop2000 (NanoDrop, Wilmington, DE, USA). The quality of RNAs were examined by using RNA Agilent Bioanalyzer 2100 (Agilent Technologies). Verified RNA samples were sent to Beijing Genomics Institute (BGI) (Beijing, China) for small RNA library preparation and sequencing. In total, nine small RNA libraries (3 for nymph antennae, 3 for male antennae and 3 for female antennae) were constructed and sequenced. In brief, total RNAs were fractionated on a 15% urea-polyacrylamide gel electrophoresis and small RNAs with corresponding bands (18–30 nt) were gel-extracted. To prepare the templates for reverse transcription, the 5-adenylated, the 3-blocked adaptor and the Unique molecular identifier (UMI)-labeled primer were added to 3′-end. 5-adoptor was added to 5′-end of purified products via T4 RNA ligase. cDNA libraries were synthesized from ligated templates and further PCR-amplified. The PCR products were gel-purified and utilized for DNA nanoballs (DNBs) generation. DNBs were read and sequenced on a DNBSEQ (BGISEQ-500) platform. The summary of experimental pipeline is given in Figure S7.

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3.3.Data processing, sequence analysis, and miRNAs identification

The clean sequences were acquired by filtering the raw reads through removing low quality tags, 5' primer contaminants, tags without 3' primer, tags without insertion, tags with poly A and tags shorter than 18 nt. Moreover, tags with copy numbers < 10 were also eliminated. The clean reads were mapped against the *A. lucorum* genome (GCA_009739505.2) and other sRNA databases using Bowtie 2 [76], and against Rfam database (http://rfam.xfam.org/) using Infernal 1.1 (CMSearch) [77] to remove tRNA, rRNA, snRNA and snoRNA sequences. To identify known miRNAs, the refined reads were locally blasted against the Hexapoda miRNAs downloaded from miRbase (Release 22.1) [67]. The unannotated clean reads (unknown sRNAs) were aligned against the reference genome and subjected to the mirDeep2 to predict the novel miRNAs. mirDeep identifies the putative novel miRNAs based on the hairpin structure of miRNA precursor and proved to be highly accurate (~ 99%) for predicting the animals miRNAs [78, 79].

3.4. Chemosensory-associated genes target prediction

A local library of chemosensory-associated genes comprising 105 odorant receptors (ORs), 24 ionotropic receptors (IRs), 38 odorant binding proteins (OBPs), 17 chemosensory proteins (CSPs), 3 sensory neuron membrane proteins (SNMPs) and 11 gustatory receptors (GRs) were created based on our previous dataset [49, 50]. If applicable, the 3' and 5' UTR regions of target genes were re-examined and extended through local blast against 3rd generation antennal transcriptome data (unpublished data) and genome sequences. Moreover, a comprehensive analysis was conducted to identify the target genes for all the miRNAs. miRanda [80] and RNAhybrid [81] softwares were utilized to determine the putative targets of known and novel miRNA identified from antennae of *A. lucorum*. The cutoff parameters for two computational prediction algorithms were set as follows: score ≥ 140 and minimum free energy (MEF) ≤ −25 Kcal/mol for miRanda; MEF ≤ −25 Kcal/mol and P-value ≤ 0.05 for RNAhybrid. The targets that tagged by both algorithms were picked as predicted targets.

3.5. Analysis of miRNAs expression and differentially expressed miRNAs

In each library, the read numbers corresponding to each known and novel miRNA were counted, and the normalized expression levels (TPM: transcript per million) were estimated by using the following formula: mapped read count / total read counts × 10^6 [82]. The variations in the expression levels of identified miRNAs between different libraries (nymph vs. adult male, nymph vs. adult female, adult male vs. adult female) were analyzed by DESeq2 R package [83]. DESeq2 employs negative binomial distribution to calculate the *P*-value. The calculated *P*-values were subjected to the Benjamini-Hochberg method to adjust the *P*-values and to evaluate the false discovery rate (FDR) [84]. The combination of adjusted *P*-value and fold change were applied to determine statistically differentially expressed miRNAs (DEmiRNAs) as follows: adjusted *P*-value ≤ 0.05 and Log2 fold change ≤ -1 for down-regulated miRNA; adjusted *P*-value ≤ 0.05 and Log2 fold change ≥ 1 for up-regulated miRNA.

3.6. Validation of DEmiRNAs expression using qPCR

To verify the accuracy and integrity of miRNA deep-sequencing data, qPCR measurements were performed on an ABI Prism 7500 Fast Detection System (Applied Biosystems, Carlsbad, CA). The same RNA samples utilized for miRNA sequencing were used to validate the expression of some DEmiRNAs. In each comparison group (nymph vs. adult male; nymph vs. adult female; adult male vs. adult female), five DEmiRNAs with highest fold change values were chosen for expression analysis. U6 snRNA (small nuclear RNA) was used as an internal reference for normalization of relative expression level of each miRNA [86]. The specific primers (Table S12) were designed by miRprimer2 [87]. Total RNAs (1 μg) were reverse-transcribed into first strand cDNAs by miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN Biotech. Co., Beijing, China). The specificities of primer pairs were assessed by melt curve analysis, and efficiencies of primers were

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assessed by 5-fold dilution series of cDNA templates. The qPCRs were conducted using miRcute Plus miRNA qPCR Kit (TIANGEN Biotech. Co., Beijing, China) in 20 μL reactions as follow: 10 μL of 2× miRcute Plus miRNA PreMix, 1 μL of cDNA (~200 ng), 0.5 μL of each primer pair (10 μmol/L) and 8 μL of nuclease-free H2O. The qPCR parameters were set as follow: 15 min at 95 °C; 20 s at 94 °C, 34 s at 60 °C (40 cycles); 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C (melt curve stages). The qPCR tests were performed with three independent biological repetitions, and three technical repeats for each biological repetition. The data were analyzed according to the comparative $2^{-\Delta\Delta CT}$ formula [88]. In each group, the differences between the expression of miRNAs were examined using Student's t-tests.

3.7. Target prediction and functional annotation of DEmiRNAs

miRanda and RNAhybrid softwares were employed to identify the candidate target genes of DEmiRNAs. The selected targets were then subjected to enrichment analysis by using the Gene Ontology (GO) (www.geneontology.org/) database. Functional pathways of target mRNAs were disclosed by The Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.jp/kegg/pathway.html) database. Finally, to clarify the regulatory interactions of DEmiRNAs and corresponding target mRNAs, miRNA-mRNA networks were built by Cytoscape software [85].

4. Conclusions

In the current work, the global miRNAs profiles from antennae of last instar nymphs, adult males and adult females of *A. lucorum* were comprehensively investigated by constructing and sequencing nine small RNA libraries. Different bioinformatics approaches were employed to identify and characterize the miRNAs expression and their putative targets. In total, 399 known and novel miRNAs were identified. A large set of genes including chemosensory-associated ones were predicted as the putative targets of identified miRNAs. These findings will assist to better comprehend the roles of miRNAs in the insects' antennae, especially chemosensation. However, advance *in vitro* and *in vivo* functional investigations are required to unravel the exact molecular regulatory mechanism of miRNA-mRNA networks in the antennae of *A. lucorum*.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplementary file 1: the list of known and novel miRNAs in the antennae of *Apolygus lucorum*; Table S1: Summary of sequencing data for each sample from the antennae of *Apolygus lucorum*; Table S2: Alignment statistics of clean tags to *Apolygus lucorum* genome; Table S3: The most abundant known miRNAs in the antennae of nymph of *Apolygus lucorum*; Table S4: The most abundant known miRNAs in the antennae of adult male of *Apolygus lucorum*; Table S5: The most abundant known miRNAs in the antennae of adult female of *Apolygus lucorum*; Table S6: The most abundant novel miRNAs in antennae of nymph of *Apolygus lucorum*; Table S7: The most abundant novel miRNAs in antennae of adult male of *Apolygus lucorum*; Table S8: The most abundant novel miRNAs in antennae of adult female of *Apolygus lucorum*; Table S9: The list of miRNAs differentially expressed in the antennal libraries of *Apolygus lucorum*: nymph vs. adult male; Table S10: The list of miRNAs differentially expressed in the antennal libraries of *Apolygus lucorum*: nymph vs. adult female; Table S11: The list of miRNAs differentially expressed in the antennal libraries of *Apolygus lucorum*: adult male vs. adult female; Table S12: Primers used for qPCR validation of miRNAs expression; Figure S1: The proportion of different types of sRNA in the antennae of *Apolygus lucorum*; Figure S2: Heatmap of Pearson correlation coefficient among different constructed small RNAs libraries from the antennae of *Apolygus lucorum*; Figure S3: GO and KEGG pathway enrichment analyses of total target genes of differentially expressed miRNAs in the antennae of *Apolygus lucorum*: nymph vs. adult male; Figure S4: GO and KEGG pathway enrichment analyses of total target genes of differentially expressed miRNAs in the antennae of *Apolygus lucorum*: nymph vs. adult female; Figure S5: GO and KEGG pathway enrichment analyses of total

target genes of differentially expressed miRNAs in the antennae of *Apolygus lucorum*: adult male vs. adult female; Figure S6: miRNA-mRNA networks of differentially expressed miRNAs in the antennae of *Apolygus lucorum* and some of their target mRNAs; Figure S7: Experimental workflow of small RNA library construction and sequencing

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