Differences in microRNAs and their expressions between foraging and dancing honey bees, *Apis mellifera* L.

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**Abstract**

Many studies have established that microRNAs (miRNAs) regulate gene expression in various biological processes in mammals and insects including honey bees. Dancing behavior is a form of communication unique to honey bees. However, it remains unclear which miRNAs regulate the dancing behavior in honey bees, and how. In the present study, total small RNAs (sRNAs) in *Apis mellifera* foragers and dancers were extracted and analyzed by a Solexa Sequencer to determine differentially expressed miRNAs. A small percentage (12.62%) of the unique sRNAs (the number of sequence types) were shared between foragers and dancers, but their expression accounted for 92.92% of the total sRNAs (the number of all sequence reads), and the length of them centered around 22 nt. Out of 58 previously identified miRNAs, 54 were present in both foragers and dancers and most of them were down-regulated in dancers. The fold-changes of *ame*-miR-34, *ame*-miR-210, *ame*-miR-278 and *ame*-miR-282 were higher than 2. 86 and 104 novel miRNAs were detected in foragers and dancers, respectively. Furthermore, two known miRNAs (*ame*-miR-278 and *ame*-miR-282) were confirmed, by qPCR, to have lower expressions in dancers. The target genes of miRNAs were substantially different between the foraging and dancing stages, and suggest that miRNAs might play important roles in regulating dancing behaviors in honey bees.

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1. Introduction

The waggle dance in honey bees (*Apis mellifera*) conveys both direction and distance information, and plays significant roles in recruiting new foragers to a food source (von Frisch, 1967; Michelsen et al., 1992). The waggle dance can also convey information about food quality (Seeley, 1995; Thom et al., 2007; Afik et al., 2008; Seefeldt and De Marco, 2008; Abbott and Dukas, 2009). In the foraging process, honey bees can integrate visual, olfactory, auditory and tactile information, and central projections of these four sensory systems involved in honey bee dance have been identified by neuronal tracer studies (Brockmann and Robinson, 2007). Recent studies have provided us with insights about the biochemical and molecular processes underlying the foraging behavior of honey bees. Octopamine is shown to initiate and maintain foraging behavior, affect the assessment of nectar profitability (Schulz et al., 2002; Barron et al., 2007; Giray et al., 2007), and enhance appetitive learning (Hammer and Menzel, 1998). Dopamine plays a vital role in honey bees foraging, learning and motor behaviors as well (Vergoz et al., 2007; Nomura et al., 2009; Mustard et al., 2010). Since the publication of the honey bee genome, behavioral studies have been carried out at genomic, transcriptomic and proteomic levels. Foraging and dancing behaviors were affected by several genes (Johnson et al., 2002; Oldroyd and Thompson, 2006). Wang et al. (2010) analyzed the expression of the peripheral insulin receptor substrate (IRS) in foraging honey bees, and found that bees with reduced IRS expression foraged more for protein-rich foods. Nicholas (2010) discovered that distinct spatiotemporal foraging memories showed different gene expression patterns, involving nerve-related genes. Brockmann et al. (2009) found that eight peptides changed dynamically in honey bees performing different foraging behaviors.

There has been a surge of interest in recent years to determine how non-coding genes affect biological processes (Jolly and Lakhotia, 2006; Mercer et al., 2009; Grillari and Grillari-Voglauer, 2006-2010) and enhance appetitive learning (Hammer and Menzel, 1998). Dopamine plays a vital role in honey bees foraging, learning and motor behaviors as well (Vergoz et al., 2007; Nomura et al., 2009; Mustard et al., 2010). Since the publication of the honey bee genome, behavioral studies have been carried out at genomic, transcriptomic and proteomic levels. Foraging and dancing behaviors were affected by several genes (Johnson et al., 2002; Oldroyd and Thompson, 2006). Wang et al. (2010) analyzed the expression of the peripheral insulin receptor substrate (IRS) in foraging honey bees, and found that bees with reduced IRS expression foraged more for protein-rich foods. Nicholas (2010) discovered that distinct spatiotemporal foraging memories showed different gene expression patterns, involving nerve-related genes. Brockmann et al. (2009) found that eight peptides changed dynamically in honey bees performing different foraging behaviors.

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2.1. Honey bee samples

Three healthy colonies of “Zhongdongda-No.1” (Chen et al., 2002), a high royal jelly-yielding breed of *Apis mellifera ligustica* kept in observation hives at Zhejiang University (30.272N, 120.191E), were used in this study. Foragers and dancers (*N* = 30 per group for both) were collected from each of three different colonies, between 11:00 and 14:00. Foragers were identified as bees returning to a colony loaded with same color and form pollen on their corbiculae, and dancers were identified as pollen foragers performing wagglng dance on combs. Each forager or dancer was lowered into liquid nitrogen with a pair of forceps immediately after collection and stored at −80°C until total RNA extraction.

2.2. Total RNA extraction and small RNA library construction

Ninety forager heads were pooled as the forager sample and 90 dancer heads as the dancer sample for RNA extraction. We used the method for total RNA extraction described by Liu et al. (2011). RNA extraction and small RNA library construction were performed.

For small RNA library construction, the total RNA was size fractionated on a 15% Tris–Borate-EDTA (TBE) urea polyacrylamide gel, and the 18–30 nt fraction was recovered. Small RNAs were eluted in 0.3 M NaCl, precipitated and washed in ethanol, then dissolved in ultrapure water. The gel-purified small RNAs were ligated to the 5′ RNA adapter with T4 RNA ligase, the ligation products were gel fractionated and purified before ligating to the 3′ RNA adapter. The final ligation products were purified and amplified by RT-PCR. The amplification products were gel fractionated and purified by electrophoresis on a 6% polyacrylamide gel in TBE buffer, and a gel slice corresponding to the amplified library was eluted in 0.3 M NaCl. Then the purified PCR products were precipitated and washed by ethanol and resuspended in nuclease-free water. Finally, the purified PCR products were sequenced on a Solexa Sequencer (Solexa HiSeq2000, Illumina, Inc., American).

2.3. High-throughput sequencing and sequence analysis

After filtering out short (<18 nt), low quality and contaminant reads, the remaining reads (clean reads) were used for statistical analysis and length distribution comparison between the foragers and the dancers. The clean reads were mapped onto the *Apis mellifera* version 4.0 genome with the Short-Oligonucleotide Analysis Package (Soap, [http://www.soap.genomics.org.cn/](http://www.soap.genomics.org.cn/)), and only perfectly matched tags were used for analysis.

To identify known miRNAs in each sample, we matched all the genome-matched reads to non-coding sequences from NCBI Genbank database (including rRNA, tRNA, snRNA and snoRNA), Rfam database (Rfam9.0), and known miRNA hairpins of honey bee in the current release miRbase (miRBase13.0).

Because one sRNA (small RNA, RNA with 18–30 nt) may have two or more different annotations, all sRNAs were annotated in the order of rRNA > known miRNA > repeat > exon > intron to ensure that every sRNA has one unique annotation. The unannotated distinct small RNAs were used to identify novel candidates (sequences that have not been identified previously) with canonical hairpin structure by “Mireap” software ([http://www.sourceforge.net/projects/mireap/](http://www.sourceforge.net/projects/mireap/)).

2.4. Confirmation of novel miRNAs

Stem-loop RT PCR was performed to confirm the existence of novel miRNAs. Reverse transcriptase reactions were employed by using the ReverTra Ace qPCR RT Kit (TOYOBO, Japan) according to manufacturer’s instructions. The miRNA-specific stem-loop primers were designed according to Chen et al. (2005). All of the stem-loop RT primers and gene-specific PCR primers are listed in Table S1. Each reaction (20 μl) containing 2 μg total RNA, 4 μl 5 × RT Buffer, 1 μl RT Enzyme Mix, 1 μM stem-loop RT primer, and nuclease-free water, was incubated in a Gradient Thermal Cycler (My Gene Series, LongGene Scientific Instruments, Co. Ltd., China) in a 96-well plate at 37°C for 15 min, 98°C for 5 min, and then 4°C for holding.

The CDNAs were diluted 1:10 to perform real-time PCR by using the THUNDERBIRD SYBR qPCR Mix (QPS-201) (TOYOBO, Japan) following manufacturer’s manuals. The PCR mixture included 10 μl THUNDERBIRD SYBR qPCR Mix, 5 μl cDNA template, 8 μM forward primer and 8 μM reverse primer and nuclease-free water added to a final volume of 20 μl, amplified in Line Gene K PCR system (Bioer Technology Co., Ltd., China) at 95°C for 1 min, followed by 45 cycles of 15 s at 94°C and 40 s at 60°C. The melting curve was drawn from 60°C to 95°C at a rate of 4°C/s, 0.5°C increase per step. The melting curve for each PCR was carefully monitored to avoid non-specific amplifications. The qPCR products were detected by electrophoresis with 3% agarose gel containing Gold View and photographed by an Electrophoresis Image Analysis System (Shanghai Peiqing Science & Technology, Co. Ltd., China). Because this experiment was meant to confirm the presence of miRNAs,
we measured only one sample per miRNA and only the presence/absence of each PCR product was noted for confirmation.

2.5. Validation of differentially expressed miRNAs

We validated the differentially expressed miRNAs between dancers and foragers by qPCR. Thirty foragers and thirty dancers were collected from three other ZND No.1 colonies; see Section 2.1 for detailed methods. The heads of foragers and dancers from three colonies were analyzed by qPCR. The qPCR method is the same as that described in Section 2.4, except that triplicates were used for each miRNA, and quantification data were obtained. All primers described in Section 2.4, except that triplicates were used for each gene, were read under the default parameters. Relative quantification of each miRNA expression was calculated by $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001), and expression in foragers was used as the baseline (i.e. expression level of each miRNA in dancers was normalized to that of foragers in each colony).

2.6. Statistical analysis

To assess the differential expression of miRNAs of 21, 22 and 23 nt and miRNAs of other lengths between dancers and foragers, the ratio of expression levels in the two groups (dancer/forager) were calculated and T-tests were used to compare the average ratio in each condition to an equal-expression ratio of 1. Differences in distribution patterns in miRNA lengths between foragers and dancers were compared by contingency table analysis, and Chi Square statistic was used.

For known miRNAs expressed jointly in the two samples, the expression of miRNAs in the two samples was normalized to the same order of magnitude. We then compared their expression differences using the method described by Xin et al. (2010). We followed Xin et al. (2010), and considered the difference between expressed miRNAs to be significant only when the absolute value of fold-change ($\log_2$ (dancer/forager)) was higher than 1, and the $P$ value was less than 0.01. In the validation of differentially expressed miRNAs, T-tests were used as described above to determine if the differentially expressed miRNAs are significantly different.

2.7. Target prediction

For miRNA target gene prediction, we downloaded full-length sequences of Apis mellifera from the NCBI UniGene database (ftp://ftp.ncbi.nih.gov/repository/UniGene/Apis_mellifera/), then used PITA program (Kertesz et al., 2007) to predict miRNA targets under default parameters. We selected and analyzed the target genes with $\text{ddG} < -10$ kcal/mol from the initial results.

3. Results

3.1. Raw data and sequence analysis

Solexa Sequencer obtained 18,368,010 and 17,966,807 reads from foragers and dancers, respectively. After filtering out short (<18 nt), low quality and contaminant reads, we were left with 15,320,541 and 15,254,864 clean reads from foragers and dancers, respectively, which were used for further analysis.

The small RNA unique reads (the number of unique sequence types) and total reads (the number of all the reads), that were shared between foragers and dancers, and that were specific to each, are presented in Table 1.

The majority of the trimmed tags in the 10–30 nt range were concentrated in 21-23 nt (Fig. 1). The percentage of 21, 22 and 23 nt length sequences in dancers was higher than that in foragers (mean ± SE of dancer/forager ratio = 1.13 ± 0.04, compared against 1, $t = 5.08, P = 0.037$), while the percentage of other length sequences in dancers was lower than that in foragers (mean ± SE of dancer/forager ratio = 0.78 ± 0.12, compared against 1, $t = -5.85, P < 0.01$). The difference in distribution pattern between foragers and dancers was further supported by a significant distribution difference between the two groups when all data were analyzed together ($X^2 = 322,737, df = 27, P < 0.0001$).

In both foragers and dancers, U was the predominant nucleotide at the first site of 20–24 nt length miRNAs in both samples, while A was the key nucleotide at other positions. Additionally, the A, U, C, G utilization preferences at each position were similar for foragers and dances (Figs. S1 and S2).

Sequence analysis showed that 6694,038 reads (43.95%) in foragers and 8385,290 reads (54.97%) in dancers mapped to the genome and the total sRNAs fell into three categories: known miRNA, rRNAeuc (including tRNAs, rRNAs, snRNAs and snoRNAs) and Unannotated sRNAs.

The distributions of sRNA among the three different categories in foragers and dancers are listed in Table 2. Fifty-four known conserved miRNAs and 58 known hairpins were identified as shared between foragers and dancers. In foragers, 1270 unique reads with 3448,464 total reads can match the known hairpins, while in dancers there were 1394 unique reads with 4949,830 total reads. No miRNAs* (complementary miRNAs) were found in our two samples (Table S2). The percentage of expression levels for each known miRNA in foragers and dancers is shown in Table S3.

3.2. Differential expressions of miRNAs between dancers and foragers

The expression levels of 54 miRNAs in dancers relative to foragers are presented in Table S4. Thirty-two of the 54 known miRNAs were significantly differentially expressed (with $P$ value < 0.01 and an absolute value of fold-change $\geq 1$). All were down-regulated in dancers. The fold-change (absolute values) of ame-miR-278,
of them yielded PCR products (Fig. 2), which indicated that miRNA sequences are shown in Table S5. PCR results showed that loop RT PCR for confirmation and quantification. All the 16 novel specific novel miRNA candidates were chosen to perform Stemers and three were specific for dancers. Eleven common and five respectively. Among these, two miRNA candidates were specific for foragers and dancers, respectively. There were 13 and 14 novel miRNAs than 2.

3.3. Prediction and confirmation of novel honey bee miRNAs

Mireap predicted 86 and 104 novel miRNA candidates in foragers and dancers, respectively. There were 13 and 14 novel miRNAs with the total reads >1000 (TPM) in foragers and dancers respectively. Among these, two miRNA candidates were specific for foragers and three were specific for dancers. Eleven common and five specific novel miRNA candidates were chosen to perform Stem-loop RT PCR for confirmation and quantification. All the 16 novel miRNA sequences are shown in Table S5. PCR results showed that all of them yielded PCR products (Fig. 2), which indicated that these novel miRNAs were expressed in both foragers and dancers.

3.4. Validation of differential miRNA expression by qPCR

Solexa sequencing results showed that most of miRNAs were down-regulated in dancers. We selected the top 4 miRNAs with significant differences in expression (the absolute value of fold-change was larger than 2) for validation by qPCR. Two known miRNAs, ame-miR-278 and ame-miR-34 was over 2 (2.3-fold, 2.2-fold, 2.1-fold and 2.1-fold respectively). Ame-miR-lab-4, 133,13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276.

3.5. Target prediction

We used the PITA program to predict the target genes of ame-miR-278 and ame-miR-282, each with thousands of targets. We selected potential targets taking both ddG < -10 kcal/mol and the related functions into consideration. High-probability targets of ame-miR-278 and ame-miR-282 are shown in Table 3. Targets of ame-miR-278 included Apis mellifera protein kinase C

![Fig. 2. Expression confirmation of novel miRNAs identified by cloning from foragers using stem-loop RT PCR.](image1)

![Fig. 3. Validation of specific down-regulated miRNA genes in dancers compared to foragers in three new colonies.](image2)

### Table 2

<table>
<thead>
<tr>
<th>Category</th>
<th>Foragers</th>
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<th>Dancers</th>
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<tr>
<td></td>
<td>Unique (%)</td>
<td>Total (%)</td>
<td>Unique (%)</td>
<td>Total (%)</td>
<td></td>
</tr>
<tr>
<td>Total sRNAs</td>
<td>1360,418 (100.00)</td>
<td>15,230,541 (100.00)</td>
<td>1081,345 (100.00)</td>
<td>15,254,864 (100.00)</td>
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<tr>
<td>miRNA</td>
<td>1270 (0.09)</td>
<td>3448,464 (26.64)</td>
<td>1394 (0.13)</td>
<td>4949,830 (32.45)</td>
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<tr>
<td>rRNAetc</td>
<td>283,211 (20.82)</td>
<td>6046,224 (39.70)</td>
<td>222,063 (20.54)</td>
<td>4249,302 (27.86)</td>
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<tr>
<td>Unann</td>
<td>1075,937 (79.09)</td>
<td>5735,853 (37.66)</td>
<td>857,888 (79.34)</td>
<td>6055,732 (39.70)</td>
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</table>

- ame-miR-210
- ame-miR-282
- ame-miR-34 was over 2 (2.3-fold, 2.2-fold, 2.1-fold and 2.1-fold respectively). Ame-miR-lab-4, 133,13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276, 133, 13b, 2, 277, 317, 10, 92a, 124, 87, 14, 252, 33, 125, 929, 276.
agers and dancers, indicating the stability of miRNA sequence structures, a factor which is important for functional maintenance (Khvorova et al., 2003; Girard et al., 2006). The first and the whole nucleotide bias of novel miRNAs were different from that of known miRNAs (Figs. S1 and S2). This indicates that these novel miRNAs have different structures from known miRNAs, and may have different functions. Total sRNAs mapped to genome could be placed in three categories, namely known miRNA, rRNA and tRNA. Table 2 shows that in foragers and dancers, the unique reads of unannotated sRNAs and rRNAs were much more numerous than those of miRNAs, while the total reads of unannotated sRNAs, rRNAs and miRNAs were almost equal. These data suggest that although miRNA unique reads only accounted for a small percentage of sRNA unique reads, they compensated for this through higher expression. Furthermore, there might be important novel miRNAs among the un-annotated sRNAs.

The following miRNAs were the 5 miRNAs with the highest expression levels (total reads) both in foragers and dancers: ame-miR-1 (69.64%), ame-miR-276 (7.87%), ame-miR-275 (4.20%), ame-miR-184 (3.14%) and ame-miR-996 (2.58%) (Table S3), and these may be associated with fundamental vital functions. Some studies found that miR-1 is involved in muscle development (Nguyen and Frasch, 2006) and the Notch pathway, which is vital for the development of the peripheral nervous system in Drosophila (Kwon et al., 2005; Wheeler et al., 2008). Two miRNAs, mir-1 and mir-133 were found to be highly conserved and co-located in adjacent gene locations in insects and vertebrates (Rao et al., 2006; Yu et al., 2008), which is consistent with our data showing that both were highly expressed in foragers and dancers. Ame-miR-276 was preferentially expressed in the olfactory lobes and the class 1 and II small-type Kenyon cells (cells involved in learning and memory in honey bees, Szymzka et al., 2008) in the honey bee brain, and ame-miR-276 and -1000 could target the neurally related genes (Hori et al., 2011). Furthermore, it has been suggested that miR-184 is involved in the central nervous system in Drosophila (Li et al., 2011). These studies, along with our results, indicate that the highly expressed miRNAs, ame-miR-1, ame-miR-276 and ame-miR-184 may be involved in neural function and perhaps in foraging and dancing behaviors.

Solexa sequencing data showed that the majority of miRNAs were down-regulated in dancers, and we further confirmed that the expression of ame-miR-278 and ame-miR-282 were lower in dancers than in foragers, suggesting that the target genes of these miRNAs are activated in dancers. Two studies revealed that in Drosophila and the silkworm, lower miR-278 expression is associated with foraging and dancing behaviors (Li et al., 2011), which is consistent with our data showing that both groups, using bees from three new colonies. The results showed that miRNAs play important roles in the process of switching from foraging to dancing. More studies are needed to discover the molecular mechanism underlying the dancing behavior in the future.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2012.08.008.

Table 3

High-probability targets of ame-miR-278 and ame-miR-282.

<table>
<thead>
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<th>miRNAs</th>
<th>Predicted targets</th>
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<tr>
<td>ame-miR-278</td>
<td>Pck, Imd, Ndufs1, Dscam, Eyg, Syt20, LOC724697, Pfrx, LOC410662, ec, mRpL2, CUBN, Blimp-1, LOC408646, Atg2</td>
</tr>
<tr>
<td>ame-miR-282</td>
<td>Pck, Syt20, LOC551139, LOC552057, LOC724697, Tie, Di, LOC552646, LOC408820, LOC409821, LOC259836, LOC724430, LOC412777</td>
</tr>
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</table>

regulate neuromuscular junction formation (Loya et al., 2009) and participate in the Wingless/Wnt pathway (Kennell et al., 2008), which is associated with brain development in Drosophila. Recently, a hypothetical miR-8 binding site was found in the honey bee Wingless gene (Hori et al., 2011). These studies strongly suggest that more miRNAs and more non-coding genes associated with foraging and dancing behaviors will be found in the future.

In our study, we tried to find differentially expressed miRNAs between foragers and dancers; naturally, these are groups of worker bees at different stages of the foraging process. In order to overcome individual differences (including colony, age, genetics and so on), we collected foragers and dancers one by one in turn from the same colony at the same time and pooled samples from three colonies together. Thus, we established two representative sRNA pools of heads from foragers and dancers, using a powerful new-generation sequencing method. These sRNA pools contained all sRNAs involving in foraging behavior and dancing behavior completely. Comparing the two sRNA pools, we found some differences in miRNAs in the two behavioral groups, and confirmed the differential expressions of several miRNAs between the two behavioral groups, using bees from three new colonies. The results showed that miRNAs play important roles in the process of switching from foraging to dancing. More studies are needed to discover the molecular mechanism underlying the dancing behavior in the future.

References


