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Next-generation small RNA sequencing for microRNAs profiling in *Apis mellifera*: comparison between nurses and foragers

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Abstract

MicroRNAs (miRNAs) are endogenous small non-coding RNAs regulating gene expression in animals and plants. To find some differentially expressed miRNAs that may be associated with age-dependent behavioural changes in honey bees (*Apis mellifera*), we applied next-generation high-throughput sequencing technology to detect small RNAs in nurses and foragers. Our results showed that both nurses and foragers had a complicated small RNA population, and the length of small RNAs varied, 22 nucleotides being the predominant length. Combining deep sequencing and bioinformatic analysis, we discovered that nine known miRNAs were significantly different between nurses and foragers ($P < 0.01$; absolute value of fold-change ≥1). Some of their target genes were related to neural function. Moreover, 67 novel miRNAs were identified in nurses and foragers. Ame-miR-31a and ame-miR-13b were further validated using quantitative reverse-transcription PCR assays. The present study provides new information on the miRNA abundance of honey bees, and enhances our understanding of miRNA function in the regulation of honey bee development.

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Introduction

MicroRNAs (miRNAs) have many important functions in plants and animals, including growth and development, cell proliferation and death and pheromone secretion. They also have a role in diseases such as cancer (Lim *et al*., 2003; Wienholds & Plasterk, 2005; Du & Zamore, 2007; Chen *et al*., 2008) and are associated with DNA/histone methylation (Zilberman *et al*., 2003; Zheng *et al*., 2007).

The honey bee (*Apis mellifera*), a highly social insect, is universally acknowledged as a model species for the study of the mechanisms and evolution of social behaviour (Robinson *et al*., 2005). It exhibits an age-related division of labour, adult worker bees carry out a nursing role when they are young, and 2–3 weeks later, they begin foraging outside (Robinson, 2002). This role transition was confirmed to be associated with gene expression in the brain (Kucharski & Maleszka, 2002; Grozinger *et al*., 2003; Whitfield *et al*., 2003; Cash *et al*., 2005). The genes '*foraging*' (Ben-shahar *et al*., 2002) and '*malvolio*' (Ben-shahar *et al*., 2004) were reported to play a key role in the behavioural transition. Previously, we have also found many genes that were significantly differentially expressed between nurses and foragers, and that may play an important role in behavioural changes (Liu *et al*., 2011). Recently, miRNA has become a popular subject of investigation, and many studies have reported functions that had previously been ignored. Bonasio *et al*. (2010) reported that cflo-mir-64 and cflo-mir-7 were up-regulated in minor and major workers of *Camponotus floridanus* respectively, and their developmentally regulated function showed their contribution to the differences among ant castes. There are still few studies on miRNAs in the field of the honey bee, but Weaver *et al*. (2007) have validated the expression of the predicted miRNAs genes in this species. Sixty-five non-redundant candidate miRNAs

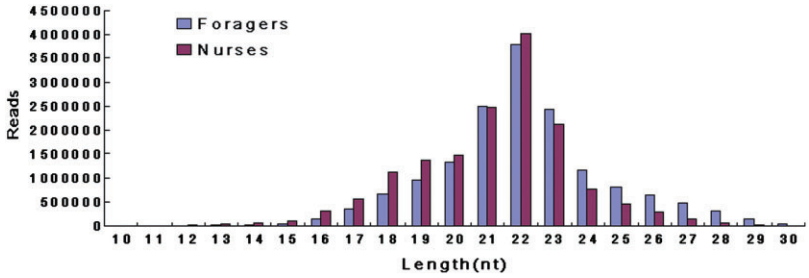

Figure 1. Length distribution of the small RNA library in nurses and foragers. nt, nucleotides.

have been identified and most of them were confirmed to be expressed in at least one honey bee tissue. Chen *et al*. (2010) analysed the repertoire of small RNAs in the honey bee from different developmental stages, and detected 267 novel miRNAs. Behura & Whitfield (2010) used quantitative real-time PCR assays and found that miRNA-124, miRNA-14, miRNA-276, miRNA-13b, let-7 and miRNA-13a were up-regulated in the young nurse bees and miRNA-12, miRNA-9, miRNA-219, miRNA-210, miRNA-263, miRNA-92 and miRNA-283 showed correlated expression patterns in old foragers. So far, there are no other reports on the relationship between miRNA and the age-dependent behavioural changes in honey bees.

In the present study, we used Solexa® sequencing (http://www.illumina.com/technology/sequencing_technology.ilmn) to detect novel miRNAs in this species using the sequenced honey bee genome as the basis of our study (The Honey Bee Genome Sequencing Consortium, 2006). We then inferred the role of miRNAs that may be associated with the age-dependent behavioural changes between nurses and foragers.

Results

Sequencing and analysing of small RNAs from nurses and foragers

Two small RNA libraries from nurses and foragers were sequenced using Solexa® technology. We obtained 17 623 540 reads and 18 368 010 reads from the libraries of nurses and foragers, respectively. After eliminating the reads of low-quality sequences, adaptor sequences, and sequences shorter than 18 nucleotides (nt), 14 332 382 and 15 230 541 clean reads of 18–30 nts in length, for the nurses and foragers respectively, remained available for analysis. The length distribution of both RNA libraries peaked at 22 nt (Fig. 1), showing 26.11% and 23.84% of total clean reads in nurses and foragers respectively. All clean reads were mapped to the honey bee genome, leading to 6 694 038 genome-matched reads (43.95%) in foragers and 6 921 188 reads (48.29%) in nurses (Table S1).

Next, all clean reads were divided into different categories of small RNAs including known miRNAs, and rRNAetc (rRNA, tRNA, snRNA, sonRNA) through mapping to the noncoding RNAs in GenBank and the Rfam database and the known miRNA miRBase 13.0 (Table 1). A significant fraction, both in nurses (37.77%) and foragers (39.70%), was RNAetc. It was found that 1239 unique tags from nurses and 1270 unique tags from foragers matched to the hairpin precursors of 58 known miRNAs, belonging to 54 families. Investigation of unique reads in both nurse and forager small RNA libraries showed that the largest fractions were unannotated small RNAs (unann) (76.10% and 79.09% of total unique clean reads respectively).

Differentially expressed microRNAs between nurses and foragers

Reads from the intersection of nurses and foragers are shown in Table S2: 92.85% of total sRNAs belong to both nurses and foragers, just 2.53% belong to nurses, and 4.61% to foragers. Ame-miR-1, ame-miR-276, ame-miR-184, ame-miR-996, ame-miR-275 are shown in Table S3 to be the five most highly expressed miRNAs both in nurses and foragers. In all, 58 miRNAs were to some extent differentially expressed between nurses and foragers, of which nine were significantly differentially

Table 1. Composition of small RNAs among different categories

	Nurses		Foragers	
Category	Unique (%)	Total (%)	Unique (%)	Total (%)
Total sRNAs	851079 (100.00%)	14332382 (100.00%)	1360418 (100.00%)	15230541 (100.00%)
sRNAs match hairpin	1239 (0.15%)	3892926 (27.16%)	1270 (0.09%)	3448464 (22.64%)
rRNAetc	202159 (23.75%)	5412967 (37.77%)	283211 (20.82%)	6046224 (39.70%)
unann	647681 (76.10%)	5026489 (35.07%)	1075937 (79.09%)	5735853 (37.66%)

unann, unannotated small RNAs.

Table 2. Significantly differentially expressed microRNAs between nurses and foragers

miR-name	fold-change(log2 nurse/forager)	*P*
ame-miR-31a	1.6610281	0
ame-let-7	1.64511019	0
ame-miR-279	1.06270156	2.07448317960418e-315
ame-miR-275	1.05412266	0
ame-miR-92a	−1.00889059	3.78E-13
ame-miR-278	−1.11600834	5.47E-13
ame-miR-210	−1.19543632	0
ame-miR-133	−1.29513352	1.39E-62
ame-miR-13b	−1.43796881	0

expressed ($P < 0.01$) and the absolute value of fold-change (≥ 1) (Table 2). Four miRNAs (ame-miR-31a, ame-let-7, ame-miR-279 and ame-miR-275) were up-regulated in the nurses and were expressed 1.66-fold, 1.64-fold, 1.06-fold and 1.05-fold more than in the foragers, respectively. By contrast, ame-miR-13b, ame-miR-133, ame-miR-210, ame-miR-278, ame-miR-92a were down-regulated in nurses and were expressed 1.44-fold, 1.29-fold, 1.19-fold, 1.12-fold, 1.01-fold more in the foragers than in the nurses (Fig. 2).

Target prediction

We used MIRANDA v3.3a (Enright *et al.*, 2003) to predict the target genes of the nine significantly differentially expressed miRNAs. As shown in Table S4, each single miRNA may target several genes, or as many as hundreds of genes, as in the case of ame-miR-31a, ame-miR-13b, ame-let-7. Of nine miRNAs, seven targeted at putative transcription factor mblk-1 (Mblk-1; NM_001011629.1), and dopamine receptor 2 (DopR2; NM_001011567.1) was modulated by ame-miR-275, ame-let-7 and also by ame-miR-278, ame-miR-210, ame-miR13b. Hyperpolarization-activated ion channel (Amih; (M_001011568.1) was the target of ame-miR-31a and ame-let-7, as well as ame-miR-278, ame-miR-210 and ame-miR-13b. Octopamine receptor (Oa1; NM_001011565.1), antennal-specific protein 3c precursor (Asp3c; NM_001011583.1) and odorant-binding protein 2 (Obp2; NM_001011591.1) are only targeted by ame-miR-278, ame-miR-31a, ame-miR-210,respectively. All three genes are related to neural function (Ebert *et al.*, 1998; Park *et al.*, 2003; Gisselmann *et al.*, 2004).

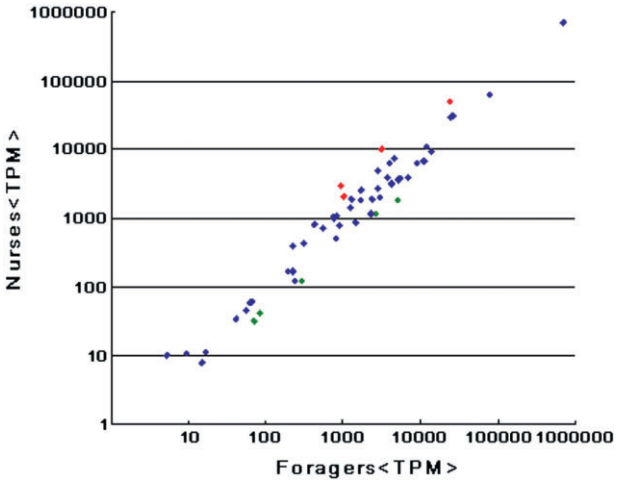

Figure 2. Comparison of microRNA (miRNA) expression levels between nurses and foragers. Note: Transcript per million (TPM) clean tags: the number of a certain tag per million clean tags, which is a normalized index. With an estimated *P*-value <0.01 and |log 2Ratio| ≥ 1(Ratio: nurses/foragers), the red shows the parts of up-regulated miRNAs in nurses; the green part represents down-regulated miRNAs, and the blue shows the part without differentially expressed miRNAs existing in both nurses and foragers.

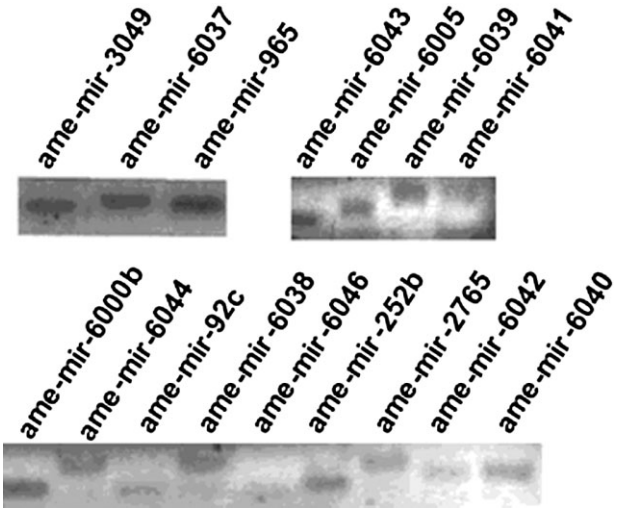

Figure 3. Expression confirmation of 16 novel microRNAs identified by cloning from nurses and foragers using stem-loop reverse-transcription PCR.

Novel microRNAs and confirmation

The representative stem-loop hairpin structure of pre-miRNA allowed us to predict novel miRNA. A total of 67 novel miRNA candidates were predicted in nurses and foragers with the inhouse software MIREAP (Table S5). Nineteen of these were selected to perform stem-loop reverse-transcription (RT)-PCR, of which, 16 yielded PCR products. This revealed that most novel miRNAs are expressed in honey bees (Fig. 3). In the across-species analysis, we found that a further 52 novel miRNAs were conserved amongst other species, and 15 were found only in honey bees.

Confirmation of primer efficiency

Specificity of quantitative RT-PCR products was documented with high-resolution gel electrophoresis and

resulted in a single product with the desired length (miR-13b, 23 bp; miR-31a, 22 bp). Additionally, melting curve analysis was performed which resulted in single product-specific melting temperatures as follows: miR-13b, 78.0°C; miR-31a, 79.5°C. No primer-dimers were generated during the 40 real-time PCR amplification cycles applied.

Real-time PCR amplification efficient and linearity

Real-time PCR efficiencies were obtained from the given slopes in LINEGENE K software (BioFlux, Tokyo, Japan). PCR efficiency (E) of each gene was calculated using the formula (Bustin *et al.*, 2009): $E = 10^{(-1/\text{slope})} - 1$. Investigated genes (miR-13b, miR-31a) showed good real-time PCR efficiency rates: for miR-13b, 1.269 in foragers and 0.933 in nurses, and for miR-31a, 2.533 in foragers and 0.996 in nurses; in the investigated range from 1.4 μg to 0.14 μg cDNA input with a high linearity (Pearson correlation coefficient $R^2 > 0.95$ [Fig. S1]).

Individual validation of sequencing data by quantitative reverse-transcription PCR

The Solexa® sequence results from pooled samples of nurses and foragers were further validated individually by quantitative RT-PCR. Sequence read analysis revealed the highest expression ratio for miR-31a and miR-13b between nurses and foragers (the copy numbers of miR-31a in nurses and foragers are 11 524 and 3228 respectively, and for miR-13b in nurses and foragers, the copy numbers are 7199 and17 278, respectively). MiR-31a and miR-13b were then selected for further individual examination by independent quantitative RT-PCR. As shown in Fig. 4A and 4B, expression levels of both ame-miR-31a ($P < 0.05$) and ame-miR-13b ($P < 0.01$) were significantly different between nurses and foragers in *t*-tests (one group). The results are consistent with the sequencing results.

Discussion

In the present study, miRNAs in nurses and foragers were identified and characterized. By comparing sRNAs in nurses with those in foragers, we found that >90% of total sRNAs correspond to <15% of unique sRNAs (Table S2). We showed that only a small number of sRNAs with high expression levels play an important role in the age-dependent behavioural changes in the honey bee from nursing to foraging. Chen *et al.* (2010) reported 267 novel honey bee miRNAs in four developmental stages (egg, larva, pupa and adult) of the honey bee, while in the present study we found 107 novel miRNAs in adult honey bees, 40 of which were identical to those reported by Chen *et al.*

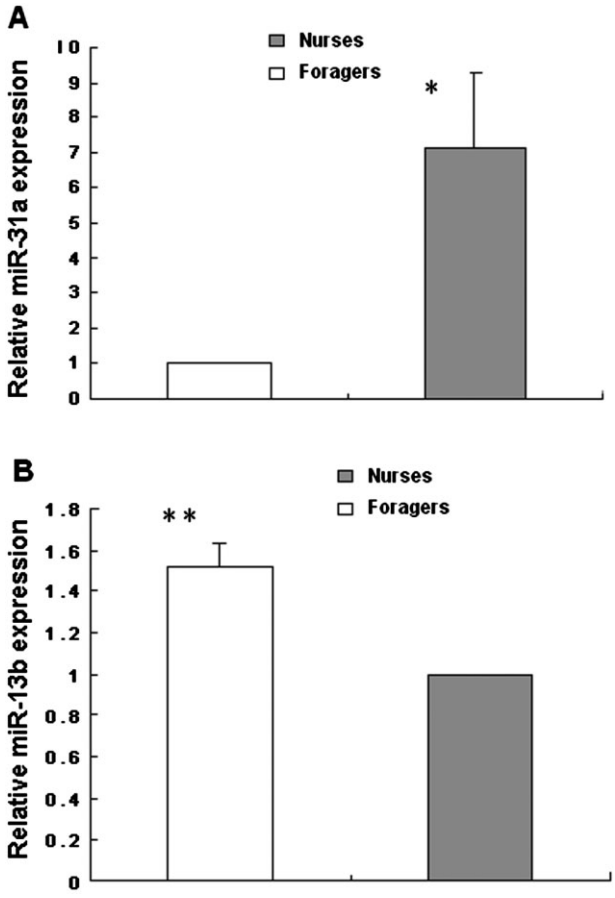

Figure 4. Individual validation of sequencing data by quantitative reverse-transcription PCR ($n = 5$, each group). Data are means ± SE. Results of *t*-test are shown (**$P < 0.01$;*$P < 0.05$) (A) Differential expression of miR-31a in nurses and foragers. (B) Differential expression of miR-13b in nurses and foragers.

Using bioinformatic analysis, we discovered nine known miRNAs were significantly different between nurses and foragers. Expression ratios of ame-miR-31a and ame-miR-13b between nurses and foragers were the highest two, and were further confirmed by quantitative RT-PCR. Ame-miR-31a was up-regulated in nurses, and ame-miR-13b in foragers. It is likely that some miRNAs play an important role in the behavioural transition from nursing to foraging. Behura & Whitfield (2010) claimed that let-7 and miR-210 were up-regulated in young nurse bees and old forager bees respectively, which is consistent with the present results. This indicates the importance of both let-7 and miR-210 in the behavioural transition from nursing to foraging. Additionally, *Apis mellifera* neuronal nicotinic acetylcholine receptor alpha7-1(NM_001011621.1; Table S4), one target of ame-miR-210, only exists in adult neuropil regions (Thany *et al.*, 2005). It is associated with learning and memory processes such as mushroom bodies and antennal lobes in honey bees (Müller, 2000; Menzel, 2001).

We found that the five most highly expressed miRNAs were ame-miR-1, ame-miR-276, ame-miR-184, ame-miR-996 and ame-miR-275, both in nurses and foragers (Table S3). Among these, ame-miR-275 showed significantly different expression between the two samples. We predicted 31 candidate target genes for ame-miR-275 (Table S4), and one of these genes is *Apis mellifera* worker-enriched antennal transcript (NM_001011562.1), which is expressed predominantly in worker antennae and legs, which has been shown to be involved in worker-preferential chemosensory signalling (Kamikouchi *et al*., 2004). The expression of ame-miR-275 was down-regulated in foragers, which inferred their target genes might be up-regulated in foragers. This inosculates the biological function of the honey bee at the forager stage. Asp3c, which was predicted to be the target gene of ame-miR-31a, has a similar function as a chemosensory protein (Briand *et al*., 2002).

We predicted 11 candidate target genes for ame-miR-278, which included Amih, DopR2 and Oa1 relating to neural function (Ebert *et al*., 1998; Park *et al*., 2003; Gisselmann *et al*., 2004). Biogenic amines (dopamine, serotonin and octopamine) have a putative widespread role as modulators of behaviour (Huber, 2005). Octopamine (OA) was confirmed to be the most strongly associated with the transition from nurses to forager; OA levels are higher in the forager brains, regardless of age (Wagener-Hulme *et al*., 1999). These findings support the hypothesis that translation of these genes are regulated by miRNAs in the honey bee brain, and may play a key role in the transition to foraging. The present findings provide new information on the miRNA abundance of honey bees, and may help us understand miRNA function in the regulation of honey bee development.

Experimental procedures

Honey bee samples and total RNA extraction

The honey bees used for the experiment were the new *Apis mellifera ligustica* variety 'Zhenongda-No.1' (ZND No.1, Ea) with a high production of royal jelly and honey (Chen *et al*., 2002). The honey bee collection and RNA extraction was carried out as described in Liu *et al*. (2011).

Construction of small RNA libraries and high-throughput sequencing

Small RNA fragments of 18–30 nt were isolated from total RNA and purified by Noves 15% TBE-Urea gel (Invitrogen, Carlsbad, CA, USA). Then, the molecular small RNAs were ligated to a 5′ adaptor and 3′ adaptor (Illumina, San Diego, CA, USA) sequentially. Subsequently, these ligation products were reverse transcribed followed by PCR amplification. The amplification products were excised from 6% TBE-Urea gel (Invitrogen). The purified DNA fragments were used for clustering and sequencing by Illumina Genome Analyzer.

Computational analysis procedure

The subsequent procedures performed with Solexa® were image recognition, base calling, filtering adaptor sequences and detecting contaminates of samples. After the basic analysis, the following bioinformatic analysis was carried out: calculating length distribution; mapping onto the *Apis mellifera* version 4.0 genome using the program Short Oligo nucleotide Analysis Package (SOAP http://soap.genomics.org.cn/); detecting differential small RNA sequences between nurses and foragers; identification of rRNA, tRNA, snRNA, snoRNA against Rfam (9.0) and NCBI GenBank database; comparing small RNA sequences with known miRNAs deposited at miRBase (miRBase 13.0); and identifying the conserved miRNAs. The reads not matching any of the above databases were marked as 'unann'.

Differential expression analysis was performed between nurses and foragers according to Audic & Claverie (1997). Firstly, the initial counts of the clean tags of each miRNA were normalized [transcripts per million (TPM)] to obtain the normalized gene expression. The miRNAs with TPM were used for further differential expression analysis. Next, the fold-change and *P*-value were calculated according to the following formulas:

$$\text{Fold}-\text{change} = \log 2\,(\text{Nurse}/\text{Forager})$$

$$p(x \mid y) = \left(\frac{N_2}{N_1}\right)^{y} \frac{(x+y)!}{x!\,y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y_{\min} \mid x) = \sum_{y=0}^{y \le y_{\min}} p(y \mid x)$$

$$D(y \ge y_{\max} \mid x) = \sum_{y \ge y_{\max}}^{\infty} p(y \mid x)$$

where x represents the observing number of tags for a gene in one library, y represents the observing number of tags for the same gene in another library, and N_1 and N_2 are the total number of tags for the two libraries respectively.

In the present study, we considered that a gene was significantly differentially expressed with a $P < 0.01$ and the absolute value of fold-change ≥ 1.

Prediction and confirmation of novel microRNAs

The 'unann' reads were for further analysis to find novel miRNAs in honey bees. There are three features in the structure of each potential miRNA precursor: highly conserved motif upstream of the hairpin precursor structure; sRNA located in two arms of the hairpin, and far from the loop; the precursor structure folded with low free energy. By folding the flanking genome sequence of small RNAs, followed by analysis of its structural features, we can identify novel miRNA candidates. Exploiting the sequence and structure features of known miRNAs and the property of deep sequencing data, we developed a new algorithm called 'Mireap' which could identify known miRNAs and novel candidates with a canonical hairpin structure and sufficiently supported by sequencing data. This algorithm comprises two dependent parts. In the first parts, candidate miRNA sites are screened out from breakpoints defined by small RNAs mapping. In the second parts, we use a minimal stringent criterion to select miRNA candidates which ensure majority of known miRNAs are recovered with only a few exceptions whose structure cannot satisfy the common features of a miRNA gene.

Stem-loop RT-PCR was used to further validate the novel miRNAs. The 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 S6. Reverse transcriptase reactions contained 148 ng of total RNA samples. NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific-Wilmington, DE. USA) was used to quantify RNA, 500 nM stem-loop RT-primer, 1 × RT buffer, 125 μM each of deoxynucleotide triphosphates (dNTPs; Promega, Madison, WI, USA), 0.01 M DL-Dithiothreitol (Invitrogen), 200 U SuperScript II reverse transcriptase (Invitrogen) and 40 U Rnase inbitior (Promega). The 20 μl reactions were incubated in a PTC-150 MiniCycler in a 25-well block at 16°C for 30 min, 42°C for 50 min, 70°C for 15 min and then 4°C for subsequent processing. The cDNAs were 1:10 diluted to perform PCR for confirmation. The PCR mixture included 2 μl cDNA, and 0.4 μM forward primers and 0.4 μM reverse primers, 1 × PCR buffer, 125 μM each of dNTPs (Promega) and 1 U Tag polymerase (TaKaRa, Tokyo, Japan). The 20 μl reactions were performed in a MyGene Series Gradient Thermal Cycler in a 96-well plate at 95°C for 5 min, followed by 35 cycles of 15 s at 95°C, 30 s for touch down from 63°C to 58°C with −0.2°C per cycle and 30 s at 72°C and then 4°C for subsequent processing. The PCR products were detected by electrophoresis with 3% agarose gel containing GoldView and photographed under ultraviolet light.

Quantitative reverse-transcription PCR of individual known microRNAs

We peformed RT-PCR to obtain cDNA, as described above. And the quantitative real-time PCR was carried out by using the two-step RT-PCR of the THUNDERBIRD SYBR qPCR system (TOYOBO, Osaka, Honshu, Japan) and was performed with a LineGeneK quantitative PCR system (Bioer Technology Co., Ltd, Osaka, Honshu, Japan). 20 μl of reaction solution [1 μl of cDNA template, 10 μl THUNDERBIRD SYBR qPCR Mix (QPS201), 0.4 μM forward primers and 0.4 μM reverse primers, and 7.4 μl of distilled water] were used for the PCR as follows: 95°C for 1 min, and 40 cycles of 15 s at 95°C and 1 min at 60°C. At the end of the run an additional melt curve step was included from 65–95°C, rising 0.5°C/s. A no template (H_2O) was included in each reaction run.18SrRNA was used as an internal control. There was no difference in 18SrRNA levels between nurses and foragers (Benshahar *et al*., 2002). All reactions were run in triplicate. After reaction, the quantification cycle (C_q) data was determined using default threshold settings and the mean C_q was determined from the duplicate PCRs. The threshold was defined using the 'best correlation' algorithm (R^2). PCR efficiency (E) of each gene was calculated via dilution row using the formula (Bustin *et al*., 2009):

$$E = 10^{(-1/\text{slope})} - 1$$

Relative quantification of miRNA expression was calculated by using the equations $2^{-\Delta\Delta Cq}$ (Livak & Schmittgen, 2001), $^{\Delta\Delta}Cq_{\text{miR-13b}} = (Cq_{\text{miR-13b}}\text{-}Cq_{18SrRNA})_{\text{foragers}}\text{-}(Cq_{\text{miR-13b}}\text{-}Cq_{18SrRNA})_{\text{nurses}}$, and $^{\Delta\Delta}Cq_{\text{miR-31a}}$ has the same equation with $^{\Delta\Delta}Cq_{\text{miR-13b}}$. All the primers used are given in Table S6. The differences in ame-miR-13b and ame-miR-31a between nurses and foragers were significant by *t*-tests (one group).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2012.01135.x

Figure S1. Determination of real-time PCR efficiency of ame-miR-31a (A) and ame-miR-13b (B) in nurses and foragers.

Table S1. Mapping to *Apis mellifera* version 4 genome (nurses and foragers).

Table S2. Reads for intersection between nurses and foragers.

Table S3. The five highest expressed miRNAs both in nurses and foragers.

Table S4. The targets of 9 significantly differentially expressed miRNA between nurses and foragers.

Table S5. Novel microRNAs in nurses and foragers.

Table S6. Sequences of stem-loop reverse-transcription primers, forward primers and reverse primers.

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