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Short communication

Identification of novel miRNAs from the microsporidian parasite *Nosema ceranae*

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ABSTRACT

Previously, six miRNAs were identified from the microsporidian parasite *Nosema ceranae*. By taking advantage of the recently updated *N. ceranae* and honey bee genome assemblies, we re-analyzed the deep sequencing datasets. Three novel miRNAs were identified, which were further validated by plasmid cloning and sequencing. The miRNAs correlated with significantly higher number of genes from the parasite than the host. Our data suggest the parasitic miRNAs are involved in self-regulation during the proliferation.

1. Introduction

As the earliest diverging group within fungus kingdom, the microsporidia comprise a large group of spore-forming, intracellular parasites, which exclusively infect animal host (Keeling and Fast, 2002; Lee et al., 2017). The genomes of microsporidian parasites are generally compact and rely on host resources for the proliferation (Desjardins et al., 2015). Infection of microsporidia has been reported to regulate the global gene expression of the hosts (Cuomo et al., 2012; Huang et al., 2016; Li et al., 2018). However, the mechanism regulating host gene expression is poorly understood. In another fungal parasite *Botrytis cinerea*, the parasite miRNAs hijacked the host's RNAi system to suppress the immune expression of the host, as a mechanism for the invasion (Weiberg et al., 2013). *Dicer* and *Argonaute* are key genes in the RNA induced silencing complex, which are involved in miRNA maturation and target mRNA degradation (Carthew and Sontheimer, 2009). Almost half of the sequenced microsporidian parasites have lost *Dicer* and *Argonaute* orthologs (Desjardins et al., 2015). Based on the genome phylogenic analysis, the *Dicer* and *Argonaute* orthologs seem to have been lost twice during the microsporidian evolution (Huang, 2018). However, *Nosema ceranae* maintains both *Dicer* and *Argonaute* orthologs (Pelin et al., 2015).

Nosema ceranae infects and proliferates in the epithelial cells of honey bee mid gut (Fries et al., 1996; Higes et al., 2007). Previously, we identified 6 miRNAs based on the first version of the *Nosema ceranae* genome assembly (Huang and Evans, 2016). Recently, the updated versions of the *Nosema ceranae* (Ncer 3.0) and honey bee (HAv3.1) genomes have been released (Wallberg et al., 2019), which facilitates a more accurate prediction of the host - parasite interaction at miRNA and mRNA levels. In this study, we first re-analyzed previous sequencing datasets based on the updated honey bee and *N. ceranae* genome assemblies to predict any novel miRNAs. Then the miRNAs were cloned into plasmids and sequenced to exclude false positive prediction. After that, the host – parasite interactions were inferred by miRNA-mRNA associations analysis.

2. Materials and methods

2.1. Novel parasitic miRNAs prediction

The miRNA sequencing reads (NCBI Bioproject PRJNA282511) were aligned to the updated *Nosema ceranae* genome assembly Ncer 3.0 (GCA_000988165.1) using miRdeep2 package (Friedländer et al., 2012). The reads showing 100% match were used to predict novel miRNAs using miRdeep2 package.

2.2. Parasite inoculation and miRNAs validation

Eighty freshly emerged honey bees were inoculated with 10^5 *N. ceranae* spores as an infection group. An additional 80 honey bees were fed with sugar water as a control group. Fifteen honey bees were collected at 5, 6, 7, 10 dpi (day post infection). The mid-gut tissues were

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Table 1
miRNAs of *N. ceranae*. All miRNAs were validated using qPCR. The qPCR products were further cloned and sequenced to confirm the accuracy of the miRNAs. All eight miRNAs were within intergenic regions.

miRNAs	Position	Mature sequence	Precursor sequence	Forward primer sequence	Reverse primer sequence
JL-1	Intergenic	uacggagaaucuuuggaauuauagg	uagaauuuggaugauauuucuaaaauacauuuuugugaauuaugaaaaucguauuuguua cagauuuaaauacggagaaucuuuggaauuauaggc	tacggagaatctttggattatagg	tacttttttttttttttttttttttgc
JL-2	Intergenic	ugcuugugaagaauuggcaacaaccu	uuugucccaucuacaacccauacuucugcuugcuuguagaaauuggcaacaaccu	tgcttgtagaaattggcaaca	ggtccagttttttttttttttaggt
JL-3	Intergenic	uaauacuuguaaaacaucacagccu	uugugagagauauuauacaauccuguaauacuugtaaaacaucacagccu	cgcagtaatacttgtaaaacatcaca	gtccagttttttttttttttaggct
JL-4	Intergenic	gggcgucuaauggaccugauccaa	auaacaaguccauuagacgccccggcgggcgucuaauggaccugauccaa	gggcgtctaatggacct	tccagttttttttttttggatca
JL-5	Intergenic	uauaugucuaaucuggguuuuggga	uauaugucuaaucuggguuuuggauuuuuuugucuuuagaggugcgucaaaaucuuaaga cacuccccaaaaaacacguuuuauagaauug	cgcagtatatgtctaatctggt	ggtccagttttttttttttccaa
JL-7	Intergenic	ggugguuuuucuacuuuuacaaa	ggugguuuuucuacuuuuacaaaucguguuauuaucgccuaauagagacgauguauugaauu uuuauaaaaguuuuuuggaucggugacc	cgcagggttgttttctac	ggtccagttttttttttttgtaaaag
JL-8	Intergenic	uuggaucaggucccauuagacgccc	uuggaucaggucccauuagacgccccgcgggcgucuaauggacuugguauuuuuuuua	gcagttggatcaggtccat	gttttttttttttgggcgtct
JL-9	Intergenic	uuaggaucuggucuaauaucugccc	uuaggaucuggucuaauaucugcccuuuagagcagauauuagaccagauuuauuuuua	gcagttaggatctggtctaatatct	ccagttttttttttttgggca

dissected individually, and five mid-gut tissues were pooled for RNA extraction with Trizol. To eliminate DNA contamination, the RNA extracts were incubated with DNAse I (Takara) for 30 min at 37 °C. Mir-X miRNA First-Strand Synthesis Kit (Takara) was used to synthesize cDNA following the manufacture's protocol. The PCR primers were designed using miRPrimer2 (Busk, 2014). Two μL of the synthesized cDNA was added to the reaction system consisted of 10 μL 2 × TB Green Premix Ex Taq II (Takara), 0.4 μL of 50 × ROX Reference Dye II (Takara), 0.8 μL of each of the forward and reverse primers (10 μM) and 6 μL double-distilled H_2O for a total volume of 20 μL. Reactions were performed using the following cycling conditions: 50 °C for 2 min, 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The generation of melting curves and gel electrophoresis was performed to verify the specificity of the qPCR product. In addition, the qPCR product was purified using purification kit (TIANDZ) and cloned into plasmid using pClone007 Versatile Simple Vector Kit (TSINGKE), which was further transferred into Trelief™ 5α Chemically Competent Cell (TSINGKE, Beijing, China). After cloning, plasmids containing positive inserts were sequenced using ABI 3730 platform.

2.3. mRNA – miRNA association analysis

Briefly, 80 honey bees were inoculated with 10^5 *N. ceranae* spores as the infection group. Additional 80 honey bees were fed with sugar water as the control group. Three replicates were performed. Ten honey bees were collected at 24 h interval from 1 dpi to 6 dpi. The mid-gut tissues were dissected and pooled for total RNA extraction with Trizol. The small RNA and mRNA were sequenced with Illumina Hiseq2000. The raw counts were normalized, and then the edgeR package was used to identify the significantly expressed genes. The *P* values were corrected for multiple comparisons with FDR using R (Robinson et al., 2010; Team, 2008). The correlation between miRNA and mRNA was analyzed using WGCNA package (Langfelder and Horvath, 2008). Detailed experimental procedures were provided in Supplementary material.

3. Results and discussion

3.1. Known and novel miRNAs

In total, 9.6 million short reads were aligned to the parasite genome assembly. Three novel miRNAs were predicted, which were named JL-7, JL-8 and JL-9. To verify the miRNAs, primers were designed for the novel miRNA, as well as the previously identified 6 miRNAs for qPCR assay (Table 1). Eight sequenced PCR products were consistent with the prediction. The infected honey bees were positive for qPCR assay and uninfected honey bees were negative for qPCR assay. One miRNA (JL-6) was not supported by either qPCR or small RNA reads alignment, suggesting that it is a false positive predication. Consequently, miRNA JL-6 was excluded from further analysis. Alternatively, it might be due to *N. ceranae* strain variance, where the previous spores were isolated from US and the current ones were isolated from China. We then used BLAST to compare the mature miRNA sequences with the genomes of *Nosema apis*, *Nosema bombycis*, *Trachipleistophora hominis*, *Vavraia culicis*, *Pseudoloma neurophilia*, *Vittaforma corneae*, *Spraguea lophii*, *Edhazardis aedis*, *Anncaliia algerae* and *Mitosporidium daphniae*, but no significant matches were found. These results suggest that the evolution of miRNA genes in microsporidian parasites has been lineage specific, which was different from the conserved *Dicer* and *Agronaute* orthologs (Desjardins et al., 2015; Huang, 2018).

3.2. Putative function of N. ceranae miRNAs

In order to infer the putative function of the 8 parasite miRNAs, the miRNA-mRNA association datasets (NCBI Bioproject PRJNA399493) were re-analyzed using updated miRNAs and genome assemblies. The parasite miRNAs were detected as early as 2 dpi and increased afterward

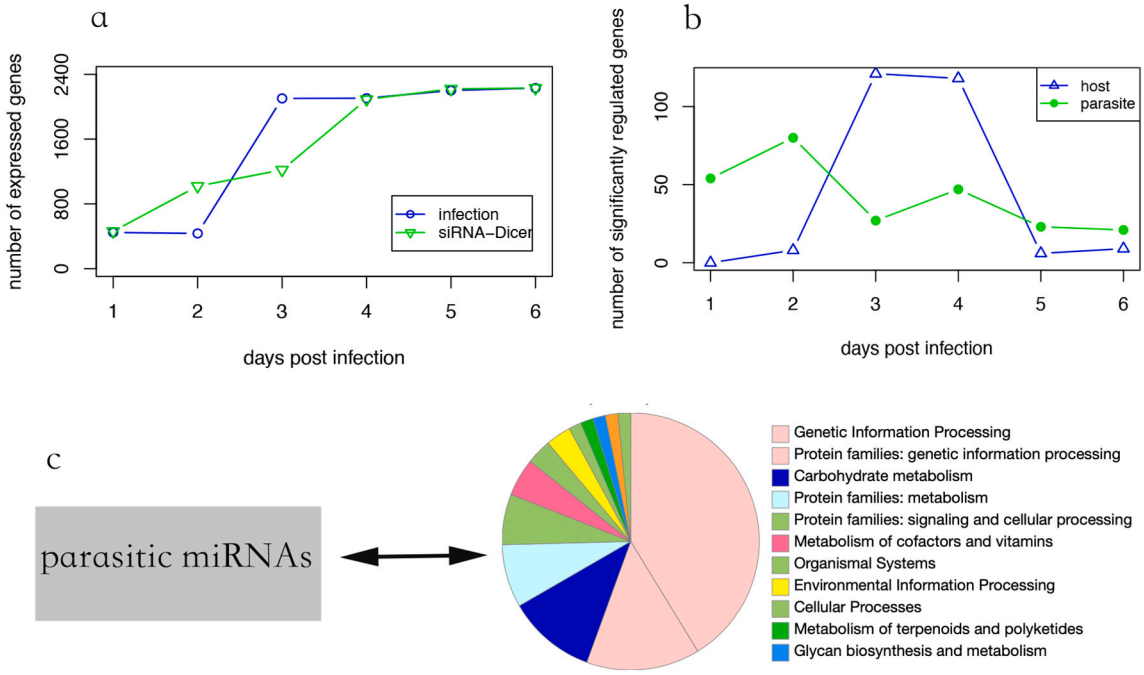

Fig. 1. The expression profile of the parasite miRNAs. (a) the number of *N. ceranae* genes expressed over time with and without *Dicer* gene suppression. Infection indicates the bees were inoculated with *N. ceranae*. siRNA-Dicer indicates the bees were inoculated with *N. ceranae* and suppressed the gene expression of *Dicer* with siRNA. It shows that the gene expression was delayed at 3 dpi when the gene *Dicer* was suppressed. (b) the number of significantly regulated genes of honey bee (host) and *N. ceranae* (parasite) over the experimental period. (c) Types of genes regulated by the miRNAs of the parasite *N. ceranae*. The 8 *N. ceranae* miRNAs correlated with 226 parasite genes, which were mainly involved in genetic information processing and metabolism.

in the infection group. When the gene *Dicer* was suppressed by siRNAs, the expression levels of miRNAs dropped at 3 dpi and then increased afterward. Additionally, a smaller number of parasite genes were expressed when the gene *Dicer* was suppressed, suggesting that the proliferation of the parasite was delayed (Fig. 1a). At 2 dpi, the parasite showed the highest number of significantly regulated genes, which then decreased steady. Comparatively, the host showed highest number of significantly regulated genes at 3 and 4 dpi (Fig. 1b). It suggests the early phase is essential to control the parasite proliferation (Huang et al., 2019). By transcriptomic analysis between the honey bees inoculated with *N. ceranae* and the honey bees inoculated with *N. ceranae* plus siRNA-Dicer, 252 honey bee genes were significantly differentially expressed during the experimental period. Out of 252 genes, 17 genes were significantly correlated with the 8 parasite miRNAs at the expression level. Comparatively, 262 parasite genes were significantly regulated, out of which 226 genes were correlated with the parasite miRNAs during the experimental period. The proportion of parasite genes correlated with parasite miRNA was significantly higher than the proportion of host genes so correlated (Fisher's exact test, $P < 0.001$). The 226 parasite genes were mainly involved in genetic information processing and metabolism (Fig. 1c). This suggests that the parasite miRNAs are involved in self-regulation during the proliferation. However, the parasite miRNAs might also interfering with the host genes, as found in other parasites (Marks et al., 2019; Weiberg et al., 2013; Zheng et al., 2013).

Data accessibility

Not applicable.

Author contribution

QH designed the experiment. SSS performed qPCR and cloning. SSS, WYY and QH organized the manuscript.

Credit author statement

QH designed the experiment. SSS performed qPCR and cloning. SSS, WYY and QH organized the manuscript. All authors approve the submitted version.

Declaration of Competing Interest

Authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104930.

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