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Comparative profiling of microRNAs and their association with sexual dimorphism in the fig wasp *Ceratosolen solmsi*

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ABSTRACT

microRNAs (miRNAs) are small noncoding RNAs that regulate various biological processes, including insect metamorphosis and sexual dimorphism. The fig-pollinating wasp, *Ceratosolen solmsi*, is a member of the super family Chalcidoidea, which have mutualistic relationships with their fig tree hosts. *C. solmsi* exhibits extreme sexual dimorphism, which corresponds to the distinct lifestyles of both sexes. Our previous studies showed that these dimorphic characteristics are associated with a dimorphic gene expression pattern. In this study, we constructed six small RNA libraries from female and male wasps in three different developmental stages, i.e., larvae, early pupae, and late pupae. We detected known miRNAs and predicted novel miRNAs, and compared their expression patterns in both sexes and among different developmental stages. We focused mainly on the miRNAs with significantly different expression patterns between sexes and among different life stages, as well as their putative associations with metamorphosis and the formation of sexual dimorphism.

1. Introduction

Sexual dimorphism is ubiquitous across insects, in which both sexes exhibit differences in behavioral, morphological, physiological traits, and/or life histories. Although this is a common characteristic among insects, extreme sexual dimorphism is rare. There is still some debate but it is easily accepted that natural selection and/or sexual selection are important for the appearance of sexual dimorphism (Kottler, 1980), in which ecological and environmental factors may induce dimorphic traits in females and males harboring highly similar genomes (Slatkin, 1984; Badyaev, 2002). Considering this similarity, most of the phenotypic differences are mediated by the sex-biased expression of genes, in which differentiation may involve a significant proportion of the genome (Ranz et al., 2003; Ellegren and Parsch, 2007). Differential gene expression depends on regulation at the transcriptional and post-transcriptional levels, and regulators such as microRNAs (miRNAs) are reported to have important roles in these processes (Marco et al., 2013; Marco, 2014).

MiRNAs comprise a class of small endogenous non-coding RNAs, which are short (ca. 22 nucleotides [nt]), single-stranded, and operate at the post-transcriptional level mostly by blocking target mRNAs via complementary pairing (Bartel, 2004) or, in some cases, stimulating translation of mRNAs (Mengardi et al., 2017). Besides miRNAs, there is another class of small non-coding RNAs, piRNAs (24–31 nt length), that accomplish their regulatory function through binding to PIWI proteins from the Argonaute family, resulting in the formation of a silencing ribonucleoprotein complex that can recognize and silence complementary sequences (Kim et al., 2009). The short length of miRNAs make them flexible in terms of spatial and temporal gene expression regulation (Flynt and Lai, 2008). Thus, miRNAs may participate in the finely tuned regulation of many biological processes in insects, including embryonic development, immunity, circadian rhythms, metamorphosis, metabolism, and sexual differentiation (Bartel, 2009; Ghildiyal and Zamore, 2009; Chen and Rosbash, 2016; Belles, 2017). Deep sequencing technology can be used to analyze the functional roles of miRNAs in the formation of sexual dimorphism and metamorphism of insects in high-throughput processes. Many studies have reported the sex-biased or life stage-specific expression of miRNAs in insects by sequencing miRNAs libraries from different sexes or life stages (Zhang et al., 2012; Fagegaltier et al., 2014; Hong et al., 2014; Huang et al.,

Abbreviations: (miRNAs), microRNAs; (GO), Gene Ontology; (KEGG), Kyoto Encyclopedia of Genes and Genomes; (TPM), transcripts per million

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2014; Liu et al., 2014; Marco, 2014; Lucas et al., 2015; Ninova et al., 2016; Peng et al., 2016).

Fig-pollinating wasps (Agaonidae: Chalcidoidea) are an insect group with extreme sexual dimorphism. They have an obligate, pollinating mutualism with fig trees (*Ficus*) (Weiblen, 2002; Cook and Rasplus, 2003), which originated about 75 million years ago (Cruaud et al., 2012). The males spend their entire lives inside figs, but the females retain a brief free-living stage of 1–2 days (Jevanandam et al., 2013). Complex selection has led to extreme anatomical sexual dimorphism. The females resemble related wasps, but they have key adaptations for entering figs, including a narrow head shape and detachable antennae. By contrast, the males have many morphological reductions, including no wings and de-pigmentation, as well as reduced eyes and antennae (Weiblen, 2002; Cook and Rasplus, 2003). In our previous studies of a fig wasp species, *Ceratosolen solmsi*, with extreme intraspecific morphological divergence in terms the compound eyes, wings, antennae, body color, and size, we found that morphological divergence occurred in the pupal stage, and there was a strong bias in the sex-differential gene expressions throughout the developmental stages of larvae, pupae, and adults, where the gene differentiation rate increased during the developmental stages (Xiao et al., 2013; Jia et al., 2014). Interestingly, throughout the developmental process, males appeared to use a drastically reduced gene repertoire, where reduced gene expression was most evident in the late pupae and adults, especially in the late pupae, and about 50% genes were downregulated in males against 5% in females. In the present study, we constructed and sequenced six small RNA libraries from each sex of *C. solmsi* during the developmental stages of larvae, early pupae, and late pupae. We identified and compared the miRNAs expressed throughout the three stages and between each sex in order to understand the roles of miRNAs with stage-specific or sex-biased expression during the formation of sex determination, sexual dimorphism, and metamorphism in *C. solmsi*, thereby helping to understand the mechanism of extreme sexual dimorphism in this fig wasp species.

2. Results

2.1. Deep sequencing of small RNA libraries in C. solmsi

To identify the sex-biased miRNAs in different stages of fig wasp development, we constructed six small RNA libraries from the whole bodies of females and males at three developmental stages, i.e., late larvae, early pupae, and late pupae, using Illumina Solexa high-throughput sequencing. At least 10 M clean reads were gained for each library (Table 1). These data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (accession number: PRJNA379062). The length distribution patterns of the six samples were compared (Fig. S1), which showed that the majority of the small RNAs ranged from 21 nt to 24 nt in length, with a peak of 22 nt, which was typical for the distribution of mature miRNAs. Samples from both genders of larvae had a peak length longer than 28 nt, which was the length range of piRNA, indicating that piRNA might be expressed in the larval stages of this fig wasp species (these putative piRNA sequences were excluded from further analyses).

2.2. Identification of known and novel miRNAs

By filtering the data set and blasting against known mature miRNAs and miRNA precursors in the miRBase 20.0 (http://www.mirbase.org), we identified 202 miRNAs and 218 miRNA precursors (Table 2, and Table S1 for more detailed information on the corresponding relationship between miRNAs and miRNA precursors). The samples from female larvae, male larvae, female early pupae, male early pupae, female late pupae, and male late pupae yielded 26, 29, 52, 48, 50, and 53 known miRNAs, respectively (Fig. S2 showed comparison of the overlapped known miRNAs among the six samples). Using the Mireap program (http://sourceforge.net/projects/mireap/) and based on the mapping of small RNAs onto the whole genome sequence of *C. solmsi*, we identified miRNAs that did not map to conserved known miRNAs, which we considered to be novel or species-specific miRNAs. Among the six samples, 200, 213, 209, 345, 222, and 162 novel miRNAs were detected in female larvae, male larvae, female early pupae, male early pupae, female late pupae, and male late pupae, respectively (Comparison of the overlapped novel miRNAs among the six samples were presented in Fig. S3).

2.3. Expression analyses of miRNAs

2.3.1. Sexual and stage-specific expression differentiation

According to the absolute expression reads, nine known miRNAs accounted for the top five highest expressed miRNAs in the six samples (Table S2). The two miRNAs *cso-miR-275* and *cso-miR-184* were shared among the top five lists in all samples. We compared the expression patterns of all the identified known miRNAs between sexes and among different development stages within the same sex. In terms of the sexual differentiation, at the larval stage, we could not detect any significant differences; at the early pupal and late pupal stages, 26 miRNAs were significantly differentially expressed, where six (*cso-miR-12*, *cso-miR-124*, *cso-miR-210*, *cso-miR-375*, *cso-miR-7*, and *cso-miR-8*) differed in both stages, nine differed only in early pupae, and 11 differed in late pupae (Table 3). When we compared the expression of known miRNAs among the different developmental stages within the same sex (Table S3), we detected 48 differentially expressed miRNAs, where 47 differed in females and 40 in males. Due to the zero (standard treated as 0.01 in the data) or low expression of many miRNAs in the larvae samples, most of the miRNAs had upregulated expression in the pupal stages (32 miRNAs were upregulated in both pupal stages in both sexes, and 11 were upregulated in only one pupal stage in one sex). Interestingly, five miRNAs (*cso-bantam*, *cso-miR-315*, *cso-miR-750*, *cso-miR-92b*, and *cso-miR-9a*) had downregulated expression in the pupal stages, where the downregulation patterns were the same in both sexes. For the predicted novel miRNAs, we also compared the differences in expression between both sexes and among the three developmental stages (Table S4), and detected that the differentially expressed novel miRNAs were more than known miRNAs. For example for the sexually differentially expressed miRNAs, in the larval stage, 44 novel miRNAs were differential, while no known miRNAs were differential; there were 95 and 107 novel miRNAs sexually differentially expressed at the early pupal and late pupal stages, respectively, while the numbers for known miRNAs were

Table 1
Clean data information on the six small RNA libraries.

Type	LarvaF count (%)	LarvaM count (%)	Pupae21F count (%)	Pupae21M count (%)	Pupae25F count (%)	Pupae25M count (%)
High quality reads	14285022 (100)	14282019 (100)	14196350 (100)	14215873 (100)	14197424 (100)	14203188 (100)
3′ adapter null	62876 (0.44)	69055 (0.48)	4210 (0.03)	4667 (0.03)	3116 (0.02)	3437 (0.02)
insert null	54749 (0.38)	46088 (0.32)	65644 (0.46)	58782 (0.41)	75922 (0.53)	88889 (0.63)
5′ adapter contaminants	72239 (0.51)	81031 (0.57)	1473958 (10.38)	1895985 (13.34)	1848590 (13.02)	2968997 (20.90)
Smaller than 18 nt	333554 (2.33)	231129 (1.62)	256525 (1.81)	207800 (1.46)	139656 (0.98)	208973 (1.47)
poly A	33 (0.00)	27 (0.00)	3737 (0.03)	2673 (0.02)	1897 (0.01)	2679 (0.02)
Clean reads	13761571 (96.34)	13854689 (97.01)	12392276 (87.29)	12045966 (84.74)	12128243 (85.43)	10930213 (76.96)

Table 2
Summary of known miRNAs numbers in each sample.

	miRNA	miRNA*	miRNA-5p	miRNA-3p	miRNA precursors	Unique sRNAs matched to miRNA precursors	Total sRNAs matched to miRNA precursors
Total known miRNA in miRBase	202	0	10	10	218		
LarvaF	26	0	2	0	30	120	211024
LarvaM	29	0	2	1	33	127	259431
Pupae21F	52	0	3	3	60	528	3812075
Pupae21M	48	0	3	1	55	484	3877487
Pupae25F	50	0	3	2	58	482	2830134
Pupae25M	53	0	3	1	60	466	2499026

miRNA: the relative abundancies clearly indicate which is the predominantly expressed miRNA; miRNA*: from the opposite arm of the precursor; miRNA-5p: data are not sufficient to determine which sequence is the predominant one and it's from the 5′ arm; miRNA-3p: from the 3′ arm; unique sRNAs matched to miRNA precursors: the number of tags with different sequences matched to miRNA precursors; total sRNAs matched to miRNA precursors: overall tags that matched to miRNA precursors.

15 and 17, respectively.

Quantitative PCR (qPCR) validation experiments were performed for five known (*cso-miR-184*, *cso-miR-12*, *cso-miR-124*, *cso-miR-275*, and *cso-miR-bantam*, in which *cso-miR-184* was used as the control for the validation of the other miRNAs) and five novel miRNAs (*novel-miR-43*, *novel-miR-128*, *novel-miR-512*, *novel-miR-337*, and *novel-miR-522*). As shown in Fig. 1, the qPCR experiments could recover the high expression of *cso-miR-275* and *novel-miR-43* as detected in the deep sequencing data. The stage-specific expression pattern of *cso-miR-bantam* and *novel-miR-128*, and the sexual differentiation of *cso-miR-12*, *cso-miR-124*, *novel-miR-128*, *novel-miR-512*, *novel-miR-337*, and *novel-miR-522* in pupal stages were also validated by qPCR experiments. The consistency between the miRNAs expression and deep sequencing data indicated a reliability of our analyses.

2.3.2. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for target genes of differentially expressed miRNAs

For known miRNAs, we first predicted all the target genes for the differentially expressed miRNAs (Table S5). Based on these target genes, GO annotation and KEGG pathway analyses were performed to identify functional modules that might be regulated by the differentially expressed known miRNAs. In all of the differentially expressed miRNAs, we could only obtain GO enrichment results for the target genes of sexually differentiated expressed miRNAs in the pupal stages. The enriched functions included heterocyclic compound binding and

Table 3
Significantly differentially expressed known miRNAs between sexes.

miR-name	Pairwise (control-treatment)	Control-std	Treatment-std	Fold-change (log_2 treatment/control)	P-value
cso-miR-12	Pupae21M-Pupae21F	8.3015	3.6313	−1.19288592	1.79E−06
	Pupae25M-Pupae25F	5.9468	2.6385	−1.17239557	0.000106554
cso-miR-124	Pupae21M-Pupae21F	22.1651	81.341	1.87569286	3.43E−97
	Pupae25M-Pupae25F	10.4298	40.0718	1.94187582	9.79E−48
cso-miR-210	Pupae21M-Pupae21F	2.4074	30.0994	3.64418695	1.94E−75
	Pupae25M-Pupae25F	2.3787	81.7926	5.10372509	1.87E−234
cso-miR-375	Pupae21M-Pupae21F	308.651	880.6292	1.51255822	0
	Pupae25M-Pupae25F	183.6195	1145.0133	2.64057317	0
cso-miR-7	Pupae21M-Pupae21F	0.01	1.9367	7.59745668	8.47E−08
	Pupae25M-Pupae25F	1.6468	0.4123	−1.99789899	0.003005873
cso-miR-8	Pupae21M-Pupae21F	29.9685	70.8506	1.2413332	4.06E−47
	Pupae25M-Pupae25F	31.3809	15.4186	−1.02521495	1.14E−15
cso-miR-190	Pupae21M-Pupae21F	1.9094	4.5189	1.24285227	0.000299156
cso-miR-2	Pupae21M-Pupae21F	3.8187	1.856	−1.04088487	0.00386124
cso-miR-278	Pupae21M-Pupae21F	1.7433	0.4035	−2.11118028	0.001178438
cso-miR-279a	Pupae21M-Pupae21F	13.4485	32.0361	1.25225326	1.76E−22
cso-miR-29b	Pupae21M-Pupae21F	0.01	1.2911	7.01245694	1.94E−05
cso-miR-305	Pupae21M-Pupae21F	2.8225	8.0695	1.5155057	1.71E−08
cso-miR-315	Pupae21M-Pupae21F	0.5811	2.0981	1.85222508	0.00109605
cso-miR-9a	Pupae21M-Pupae21F	4.3998	23.8858	2.44064335	8.25E−41
cso-miR-iab-4	Pupae21M-Pupae21F	0.6641	2.0981	1.65961104	0.002474588
cso-let-7	Pupae25M-Pupae25F	9.6979	3.8753	−1.32336438	4.97E−08
cso-miR-1	Pupae25M-Pupae25F	7241.5789	2338.0138	−1.63102084	0
cso-miR-193	Pupae25M-Pupae25F	3.3851	11.4609	1.75944998	4.62E-13
cso-miR-279b	Pupae25M-Pupae25F	0.5489	6.4313	3.55049514	9.78E-16
cso-miR-3049-3p	Pupae25M-Pupae25F	0.2745	2.3087	3.07220266	9.57E-06
cso-miR-3049-5p	Pupae25M-Pupae25F	4952.9684	13,070.566	1.39995629	0
cso-miR-33	Pupae25M-Pupae25F	9.6064	0.1649	−5.86433258	1.40E-31
cso-miR-3477	Pupae25M-Pupae25F	24.2447	9.0697	−1.41854267	8.38E-20
cso-miR-3791	Pupae25M-Pupae25F	2.0128	17.8921	3.15204699	1.88E-37
cso-miR-92a	Pupae25M-Pupae25F	78.1321	187.8261	1.26541026	1.08E-117
cso-miR-932	Pupae25M-Pupae25F	0.183	1.0719	2.55025477	0.007747149

Notes: miR-name: the name of known miRNAs in the *C. solmsi*; pairwise(control-treatment): the pairwise comparison of miRNA expression between different samples, with the former setting as the control and the latter setting as the treatment; control-std.: the normalized expression level of the control sample as transcripts per million (TPM = (miRNA total tags/total clean tags) $\times 10^6$); treatment-std.: the normalized expression level of the treatment as transcripts per million (TPM = (miRNA total tags/total clean tags) $\times 10^6$); fold change(log_2 treatment/control): log_2 ratio comparison between the treatment and control; P-value: P-value calculated from the normalized expression levels. The table only listed the significant differences with fold changes > 1 or less than -1 and P-values < 0.01.

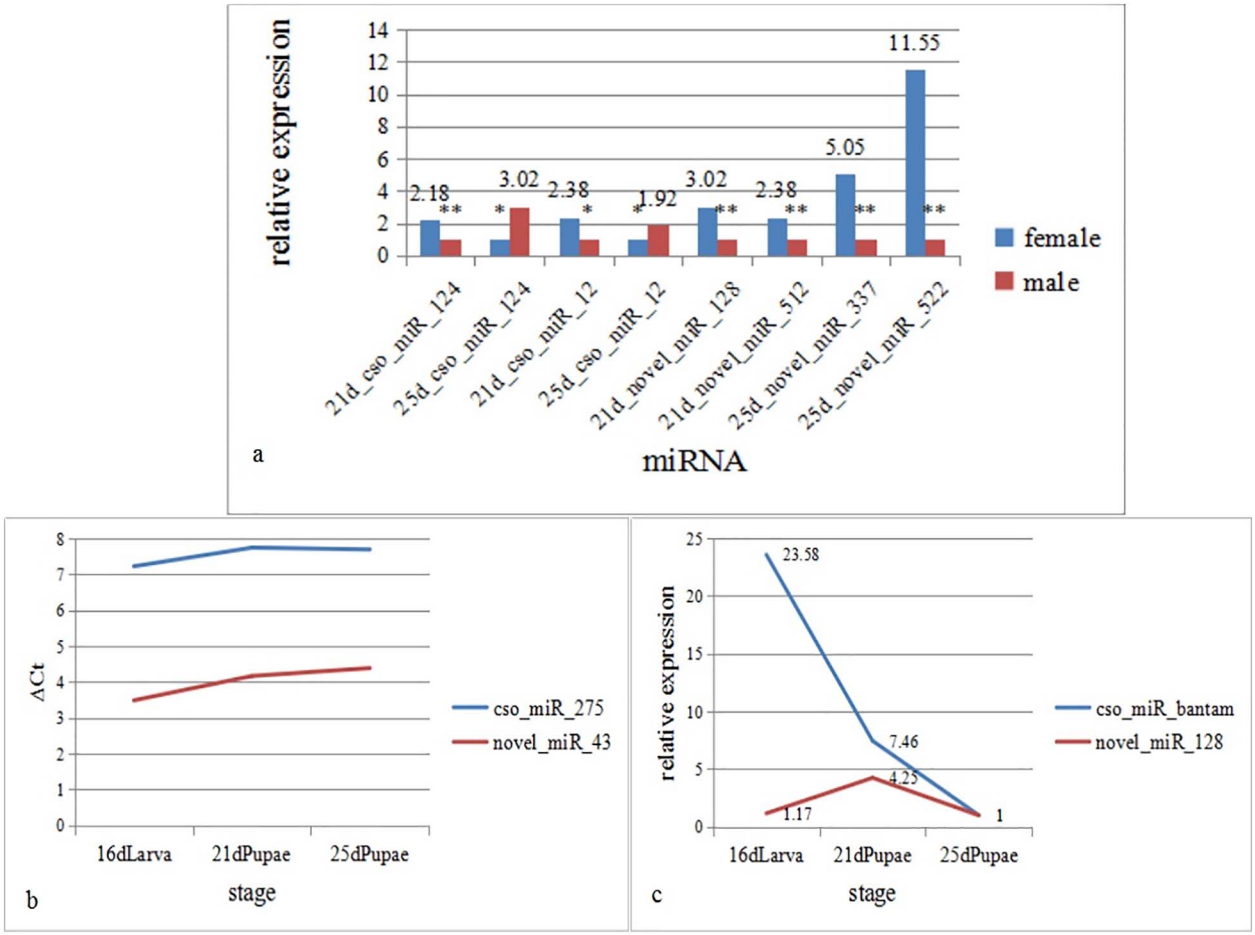

Fig. 1. Real-time quantitative PCR validates expressions of four known and five novel miRNAs. The amount of expression was normalized to the level of cso-miR-184. a) Sex-biased expression of miRNA in early pupal and later pupal stages. The bars show relative expressions and asterisks (* or**) indicate significant differences ($P < 0.05$ or $P < 0.01$, respectively) compared to the relevant control in a two-tailed *t*-test. b) The miRNAs of high expression through larval, early pupal and later pupal stages. c) The known miRNA (miR-*bantam*) shows downregulated expression pattern in pupal stages and the novel miRNA (novel_miR_128) shows upregulated expression only in early pupal stage.

organic cyclic compound binding in the early pupal stage, and developmental process and single-organism process in the late pupal stage. No KEGG pathways were enriched for the target genes of these differentially expressed known miRNAs.

For the differentially expressed novel miRNAs, we also predicted the target genes (Table S6), and GO enrichment analyses were demonstrated on these target genes. We detected that genes involved in many functional processes might be sexually differentially regulated by novel miRNAs during any developmental stage, or be differentially regulated among different developmental stages in the same sex (Table S7). In these enriched GO processes, we detected two interesting items that might be involved in the metamorphosis of the *C. solmsi*: 1) "imaginal disc-derived wing morphogenesis" between both sexes of larvae; 2) "phagocytosis" between larvae and early pupae in both sexes. For KEGG enrichment analysis, we could only detect an enrichment pathway of "Hippo signaling pathway" for the target genes of the differentially expressed novel miRNAs between both sexes of the larval stage.

3. Discussions

The characteristic roles of miRNAs in the regulation of cellular processes have attracted much interest and they have been described in diverse organisms. In insects, miRNAs can regulate developmental processes, and thus sexual differentiation and metamorphosis, via either the degradation of target mRNAs or inhibition of the translation (Friggi-Grelin et al., 2008; Bejarano et al., 2010; Rubio and Belles, 2013; Fagegaltier et al., 2014; Peng et al., 2016; Belles, 2017). In the present study, based on our previous transcriptomic comparisons of females and males in different developmental stages (larvae, early pupae, late pupae, and adults) of a fig wasp species, *C. solmsi*, which exhibits extreme sexual dimorphism (Xiao et al., 2013), we obtained the miRNAs in both sexes during three developmental stages (larvae, early pupae and late pupae, throughout the formation period of metamorphosis) and determined the sex-biased or developmental stage-specific expression of miRNAs.

3.1. Expression of miRNAs and mRNAs in both sexes of C. solmsi

When we compared the miRNAs and transcriptomes, it was interesting that the sexual differentiation patterns mapped well throughout all three developmental stages, according to the comparison between the number of sexually differentially expressed miRNAs and the proportion of sex-specific expressed genes in transcriptomic data.

For the known miRNAs, in the larval stage, no miRNAs with significant differences in expression was detected and the proportion of differentially expressed genes was only 16%. In the early pupal stage, there were 15 known miRNAs with sex-specific differences in expression, where the target genes were enriched for functions such as heterocyclic compound binding and organic cyclic compound binding. The proportion of mRNAs with sex-specific differences in expression was 19% and genes upregulated in females were enriched for terms such as channel, receptor, transmembrane, and transporter activities. The late pupal stage had the most distinct sex-specific differences in known miRNAs expression, with 17 sex-biased miRNAs and the target genes of

these miRNAs were enriched for GO terms such as developmental process and single-organism process. For the mRNAs in the late pupal stage, the proportion of genes with sex-specific differences in expression was up to 53%, among which 48% were downregulated in males; all of these sexually differentially expressed genes were enriched for GO functions involved in translation. These comparisons indicate that in the late pupal stage at least, known miRNAs may play roles in sexual dimorphism by downregulating the translation of genes in males.

For the novel miRNAs, the numbers of sexually differentially expressed miRNAs were 44, 95, and 107 in the stages of larvae, early pupae, and late pupae, respectively. GO enrichment analyses for the target genes of the sexually differentially expressed novel miRNAs detected that unlike the results for known miRNAs, these target genes' functions were enriched in many biological processes, mainly associated with the cellular component of membrane (Table S7).

To get better dialog between the sexual differential expression of miRNA and their target mRNAs in a specific developmental stage, we also provided the expression profiles of those sexual differentially expressed miRNAs and their possible target mRNAs that might be regulated in the stages of larvae, early pupae, and late pupae (Table S8). That is, if the miRNA was upregulated in male, the target mRNA was downregulated and vice-versa: if the miRNA was downregulated in male, there was an upregulation of the corresponding mRNA. For these target mRNAs, the gene functions of their homologs in public database (if available) were also provided.

3.2. Associations of miRNAs with sexual dimorphism in C. solmsi

At present, the traits of fig wasps have not been fully modeled, so it is difficult for us to investigate the roles of specific miRNAs in *C. solmsi*. However, based on the expression data and the reported functions in other insects, we can consider some of the putative functions of certain miRNAs in *C. solmsi* to some extent.

The functional enrichments of target genes for all the differentiated expressed miRNAs show that some of the known miRNAs and novel miRNAs may contribute to metamorphism and sexual dimorphism in *C. solmsi*. For example, as we have mentioned in the Results section, there are two enriched GO pathways for the target genes of differentially expressed novel miRNAs, including “imaginal disc-derived wing morphogenesis” between both sexes of larvae and “phagocytosis” between larvae and early pupae in both sexes. Thus, novel miRNAs sexually differentially expressed in the larval stage may contribute to the sexually dimorphic morphogenesis of wing in this fig wasp species. Some novel miRNAs may play roles in the process of metamorphosis from larvae to pupae via regulating genes in the biological function of phagocytosis in both sexes (Tracy and Baehrecke, 2013).

The distinct sex-biased known miRNAs are conserved and they have important roles in behavior and development in other insects. When we compare the known miRNAs expression between both sexes in *C. solmsi*, six miRNAs (*cso-miR-12*, *cso-miR-124*, *cso-miR-210*, *cso-miR-375*, *cso-miR-7*, and *cso-miR-8*) are significantly differently expressed in both pupal stages. Interestingly, the significance of these miRNAs for differentiation does not depend on zero expression in either sex, which shows that these miRNAs are important for both sexes in *C. solmsi*. The function of *miR-124* has been studied extensively in *Drosophila*, where it regulates diverse aspects of rhythmic behaviors, as well as controlling neural development, reproductive success, and transformation in sexual dimorphism (Weng et al., 2013; Wang et al., 2014; Garaulet et al., 2016; Zhang et al., 2016). In our present study, we can detect that several possible target genes of *miR-124* are microtubule associated (eg. *CSO_004598*: tubulin alpha 1b, *CSO_004015*: tubulin alpha 2, and *CSO_002766*: tubulin beta.); considering that microtubules assembled from heterodimers of alpha- and beta-tubulin are important structural component of cytoskeleton that are critical for neural cells, the sexual differentiation of its expression in the *C. solmsi* may be associated with sexual dimorphism in neural system (Minoura, 2017). *miR-7* contributes to the control of *Drosophila* wing growth (Aparicio et al., 2015), while *miR-8* has been reported to control innate immune homeostasis (Choi and Hyun, 2012) as well as growth and development in insects (Choi and Hyun, 2012; Jin et al., 2012; Loya et al., 2014; Lee et al., 2015). *miR-8* can control synapse structure during neural development as well as regulating body size in response to steroid signaling. Studies of the function of *miR-210* and *miR-375* have mainly been related to human cancers where the former can act as a fine tuner during the hypoxic response (Ivan and Huang, 2014; Yan et al., 2014). The role of *miR-12* has been investigated rarely, but a recent study in the mosquito has showed that it is critical for the persistence of *Wolbachia* in the host cells (Osei-Amo et al., 2012). According to these previous studies and the high but significant differences in the expression of these known miRNAs in both sexes, we suggest that they may be important for sexual dimorphism development during the pupal stage in *C. solmsi*, though further experimental functional validation is required.

The known miRNAs with downregulated patterns from the larval to pupal stages have strong signals associated with development and metamorphosis in *C. solmsi*. Most of the miRNAs have upregulated expression patterns from the larval to late pupal stages, except five miRNAs that have downregulated patterns (*cso-miR-315*, *cso-miR-750*, *cso-miR-92b*, *cso-miR-9a*, and *cso-bantam*, the first three of which are downregulated in both sexes and the latter two are downregulated only in males). In these miRNAs, *miR-9a* is also highly expressed in early embryos in the honeybee (Zondag et al., 2012); in *Drosophila*, it has been reported that *miR-9a* functions in developmental processes, such as in the precise specification of sensory organ precursors and preventing apoptosis during wing development (Li et al., 2006; Bejarano et al., 2010). *bantam* is highly conserved in arthropods and it has essential functions in growth, apoptosis, germline stem cell, and circadian rhythm (Smibert and Lai, 2010; Belles, 2017), while it may also be important in the immune reaction (Etebari et al., 2013; Kaewkascholkul et al., 2016). Thus, because *miR-9a* and *bantam* have roles in wing and pupa formation, we suggest that they may also participate in metamorphosis and sexual dimorphism in *C. solmsi*.

Furthermore, when we looked deeper into the putative regulation correlation of miRNAs and target mRNAs (as described in Table S8), we noticed an interesting phenomenon—the extremely sexual differentially expressed genes (with big fold change values) were often regulated by more than one miRNAs. For example, the gene encoded tubulin ahpha 2 (*CSO_004015*) mentioned above was sexually differentially expressed at early and late pupal stages with fold changes of 6.526843091 and 6.909764835 respectively. In early pupal stage, it might be regulated by six miRNAs (*ame-miR-7*, *ame-miR-8*, *ame-miR-305*, *ame-miR-279a*, *ame-miR-315* and *ame-miR-124*); in late pupal stage, it might be regulated by *ame-miR-279b* and *ame-miR-124*. Two genes (*CSO_011123* and *CSO_007018*) with sexual expression differentiation at only larval stage might also be targeted by several miRNAs. The gene *CSO_011123* (fold change: − 7.520532194), encoding metabotropic glutamate receptor, which might play roles in regulating activity-dependent synaptic facilitation and fine synaptic morphology (Bogdanik et al., 2004), had six correlated miRNAs (*ame-miR-2765-5p*, *ame-miR-993*, *novel-miR-206*, *novel-miR-290*, *novel-miR-295* and *novel-miR-296*). The gene *CSO_007018*, encoding a haem peroxidase that could catalyze the formation of covalent bonds between biomolecules and thus might function in forming extracellular and cuticle structure (Hurd et al., 2015), was also under the regulation of four miRNAs (*ame-miR-993*, *novel-miR-295*, *novel-miR-316* and *novel-miR-318*). Taken together these examples can give us a indication that except the unavoidable presence of false positives, when a gene is crucial for development (for example, *CSO_004015* and *CSO_011123* function in the morphological formation in neural system, and *CSO_007018* functions in formation of extracellular and cuticle structure), it is often regulated by several miRNAs, so if one of them is disrupted, the other ones can act in a similar way. This one-to-more correlation pattern of mRNAs and

miRNAs further means that these genes may be key genes for sexual dimorphism or other important functions in the fig wasp.

In summary, our analyses based on the miRNAs in six small RNA libraries identified many candidate miRNAs, some of which may be associated with metamorphosis and sexual dimorphism in the fig wasp *C. solmsi*. Even though functional validations of the sexual differences in miRNA expression here presented are required, our findings provide new insights for a better understanding of extremely sexually dimorphic fig wasps.

4. Materials and methods

4.1. Preparation of specimens of the fig wasp C. solmsi

The fig wasps could not be reared in the laboratory, so we collected all the samples from the field in Danzhou (19°30′N, 109°31′E), Hainan, China, during June to August 2013 (when the rainy season occurs in this area). We bagged the fig syconia with fine-mesh nylon bags in the "pre-female phase" stage before any wasps visited, and then introduced one to three mated *C. solmsi* females (the mother wasps) to each male fig in the "male phase" stage when it was receptive for oviposition by *C. solmsi*. According to our previous study, the maturation period of *C. solmsi* in the rainy season lasts for 28 (males) to 29 (females) days after the introduction of the mother wasps (Jia et al., 2014). We defined the day of introduction as day 0 when the mother wasps laid their eggs, and we dissected the figs to collect the wasps on days 16, 21, and 25 when the wasps were in the larvae, early pupae, and late pupae stages, respectively. In total, six samples types were collected: female larvae, male larvae, early female pupae, early pupae male, late female pupae, and late male pupae. In the larvae stage, when the sexes could not be identified morphologically, for each specimen, we identified the sex by PCR analysis of sex-specific transcripts of *doublesex*, which is an important gene for sex determination (Jia et al., 2014). The samples were collected and transferred immediately into 1.5-mL microcentrifuge tubes, and then stored at − 80 °C. For each sample, at least 10 specimens were used for RNA extraction.

4.2. RNA isolation, small RNA library construction, and Illumina sequencing

Total RNA was isolated from pooled wasp samples using Trizol reagent (TransGen, Beijing, China) according to the manufacturer's instructions. The quantity and quality of the total RNA samples were examined using 2100 Bioanalyzer system (Agilent, Santa Clara, USA).

Small RNA libraries were generated from the six samples with the TruSeq Small RNA Sample Prep Kits, according to the manufacturer's instructions (Illumina, CA, USA). The 18–30 nt RNA fragments were appended with 3′ and 5′ adapters, reverse transcribed, and PCR amplified. The amplified PCR products were purified and sequenced using the Illumina Hiseq2000 se50 platform (BGI, Shenzhen, China).

4.3. Standard bioinformatics analysis

The 50-nt sequence tags obtained from HiSeq sequencing were subjected to a data cleaning step, which included eliminating low quality tags (with N bases in the first 30 nt, or with more than four bases whose Phred quality score was lower than 10, or with more than six bases whose Phred quality score was lower than 13), eliminating abnormal tags (with 5′ sequencing primer, or without 3′ sequencing primer, or with poly A), and trimming 3′ sequencing primer, so that only tags with longer than 18 nt insertion were used for subsequent analyses.

The clean tags were aligned to published mRNAs sequences, RFAM, and Repbase to identify all the possible mRNAs, rRNAs, tRNAs, snRNAs, snoRNAs, and others using BLAStALL (-p blastn -F F -e 0.01) (Ruby et al., 2006). The remaining clean tags were aligned to the miRNA sequences in miRBase, and the matched or one mismatch sequences with the miRNAs of the honeybee *Apis mellifera* were identified as known miRNAs. RNAs that could not be annotated were used to predict novel miRNAs with the Mireap program (-A 18 -B 26 -a 20 -b 24 -c 5 -u 20 -e -18 -d 35 -p 14 -v 4 -s 5 -f 10) developed by the BGI (BGI, Shenzhen, China).

4.4. Comparison of differentially expressed miRNAs

The miRNAs expression patterns were compared in the females and males in different stages, i.e., larvae, early pupae, and late pupae, as well as between different stages in the female and male sexes, in order to identify sex-biased or stage-specific differentially expressed miRNAs. The transcripts were normalized to get the expression levels as transcripts per million (TPM = (miRNA total tags / total clean tags) × 10^6), where the normalized expression of 0 was modified to 0.01. We then calculated the fold changes and P-values from the normalized expression levels, and generated a $\log_2$ ratio plot and scatter plot. In the pairwise analysis of samples in this study, we only considered fold change (log2) > 1 or fold change (log2) < -1, and P-value < 0.01 as significantly differently expressed.

4.5. qPCR validation of several miRNAs' expression

The expression profiles of four known miRNAs and five novel miRNAs were investigated in this study. The miRNAs of fig wasps were isolated using EasyPure® miRNA Kit (Transgen, Beijing, China), according to the manufacturer's instructions. Reverse transcription reactions for mature miRNAs were conducted with three biological replicates total RNA (same RNA sources that were used for the sequencing experiments) using TransScript® miRNA First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China), according to the manufacturer's instructions. The Reverse transcription reactions were incubated in a Veriti Thermal Cycler (ABI, USA) for 60 min at 37 °C for 5 s at 85 °C, and then at 4 °C for subsequent processes. The reverse transcription products were used for real-time qPCR. All primers for qPCR are listed in Table S9. qPCRs were performed using TransScript® Green miRNA Two-Step qRT-PCR SuperMix following the manufacturer's instructions in a real-time thermal cycler (Mx3000P, USA). The qRT-PCR program was as follows: 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 34 s. Three biological and technical replicates were performed. Real-time expression data were analyzed by $2^{-\Delta\Delta Ct}$ methods.

4.6. mRNA target prediction and pathway analysis

Target gene prediction was performed using two softwares Targetscan (Lewis et al., 2005) and miRnada (Enright et al., 2003). The rules used were based on those suggested by Allen et al. (2005) and Schwab et al. (2005). The parameters were as follows: no more than four mismatches between an sRNA and target (G-U bases counted as 0.5 mismatches); no more than two adjacent mismatches in an miRNA/target duplex; no adjacent mismatches in positions 2–12 in an miRNA/target duplex (5′ of miRNA); no mismatches in positions 10–11 in an miRNA/target duplex; no > 2.5 mismatches in positions 1–12 in an miRNA/target duplex (5′ of miRNA); and minimum free energy (MFE) of the miRNA/target duplex ≥ 75% of the MFE of the miRNA bound to its perfect complement. The detailed parameters settings were also provided: para_targetscan = -class fly -tax 7227 -sc 0 -p 0; para_miRanda = -sc 140 -en 0 -scale 4 -strict -go -4 -ge -9 –quiet miRanda_filter = -e -20 -s 15. If a gene was detected twice to be a target gene of an miRNA, it will be considered to be confident. The GO terms and KEGG pathways were analyzed for the differentially expressed miRNAs' target genes. First, we mapped all target gene candidates onto GO terms in the database (http://www.geneontology.org/) or KEGG database (http://www.genome.jp/kegg/), where we calculated gene numbers for each term, before performing a hypergeometric test to

identify significantly enriched GO terms or KEGG pathways among the target gene candidates compared with the reference gene background. GO terms or KEGG pathways with corrected P-value ≤ 0.05 were defined as significantly enriched among the target gene candidates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.08.030.

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