Identification and Characterization of Sex-Biased MicroRNAs in *Bactrocera dorsalis* (Hendel)

Wei Peng, Kaleem Tariq, Junfei Xie, Hongyu Zhang*

State Key Laboratory of Agricultural Microbiology, Key Laboratory of Horticultural Plant Biology, Ministry of Education and Institute of Urban and Horticultural Entomology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, People’s Republic of China

* hongyu.zhang@mail.hzau.edu.cn

Abstract

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that regulate various biological processes including sexual dimorphism. The oriental fruit fly *Bactrocera dorsalis* is one of the most destructive agricultural insect pests in many Asian countries. However, no miRNAs have been identified from the separate sex and gonads to elucidate sex gonad differentiation in *B. dorsalis*. In this study, we constructed four small RNA libraries from whole body of females, males (except ovaries and testes) and ovaries, testes of *B. dorsalis* for deep sequencing. The data analysis revealed 183 known and 120 novel miRNAs from these libraries. 18 female-biased and 16 male-biased miRNAs that may be involved in sexual differentiation were found by comparing the miRNA expression profiles in the four libraries. Using a bioinformatic approach, we predicted *doublesex (dsx)* as a target gene of the female-biased miR-989-3p which is considered as the key switch gene in the sex determination of tephritid insects. This study reveals the first miRNA profile related to the sex differentiation and gives a first insight into sex differences in miRNA expression of *B. dorsalis* which could facilitate studies of the reproductive organ specific roles of miRNAs.

Introduction

Sexual dimorphism is prevalent in insects, and it is thought that, transcriptional and post-transcriptional regulators have an impact in this biological process [1,2]. The regulation of sex-biased genes expression between males and females through several classes of small RNAs play important role in the sexual differentiation [3–5]. MicroRNAs (miRNAs) are a class of approximately 22 nucleotide (nt) endogenous non-coding small RNAs generated by Dicer enzymes processing of hairpin precursors that are involved in regulation gene expression at the post-transcriptional level [1,6–8]. In insects, mature miRNAs are loaded with an Argonaute protein to repress mRNA transcript or protein translation by binding to the 3’ UTR region of the target gene with imperfect complementarity [7]. The seed region (bases 2–8 from the 5’ end), which is the most highly conserved sequences of the miRNA contributes significantly to miRNA-
target interaction [9–11]. The complex relationship between miRNA and mRNA form an intricate epigenetic mechanism for spatial and temporal gene expression regulation [12,13]. Thus miRNAs participate in regulating various biological processes including embryonic development, sexual identity, metamorphosis, fat metabolism and immune [3,14–17].

Recently, application of deep sequencing technology and in silico analysis have revealed amounts of miRNAs in plants, animals and viruses. The establishment of small RNA libraries of different life stages and specific tissue in these species stimulate a comprehensive and more in-depth studies of miRNAs in the development and other physiological process. The expression profiles and sex-biased miRNAs in sexual dimorphism have been characterized in the animals of mouse, chicken [18,19] and insects including Drosophila melanogaster, Tribolium castaneum, Manduca sexta [3,4,20,21]. Different miRNA expression profiles between testis and ovary in mouse have found 49 and 48 miRNAs exclusively existed in the gonad respectively. Mir-17-92 and Mir-106b-25 are involved in the mice spermatogenesis [18]. In D. melanogaster, sex-biased miRNAs are connected with the reproductive function and some new miRNAs are preferentially expressed in testis [4]. Furthermore, let-7 miRNA was found as a somatic systemic signal modulators to establish and maintain sexual identity in sexes and differentiation in gonads [3]. In the nondrosophilid insect T. castaneum, oocytes miRNAs play important role in maternal transcript degradation process [21]. All these diverse miRNAs between sexes highlight the significant function role in germline development and sexual differentiation.

Bactrocera dorsalis (Hendel) (the oriental fruit fly), which is a badly invasive pest because of the damage to masses of fruits and vegetables like the citrus and guava, spread all over the South-East Asia and a number of Pacific Islands [22]. The fully sequenced genome and transcriptome analyses provide a greatly foundation database to understand the genetic network and molecular mechanism in B. dorsalis [23,24]. Although the previous report have sequenced the miRNAs during different developmental stages in life cycle of B. dorsalis [25] and different developmental stages of B. dorsalis testes [26], the miRNAs functions in sex determination and differentiation remain largely unknown.

In the present study, we constructed and sequenced four small RNA libraries between each sex and gonads of mature adult B. dorsalis, and identified numerous known and novel miRNAs highly expressed in the gonads, in order to understand these sex-biased miRNAs roles in reproduction and regulation of sex determination in B. dorsalis. These specific expression data will provide new informations to elucidate the regulatory role of miRNAs in sexually dimorphic traits and might contribute to understanding the sex determination mechanism in B. dorsalis.

Results

Global analysis of small RNA libraries

To identify the sex-biased miRNAs in B. dorsalis, four small RNA libraries from the mature females, males (without ovaries and testes) and ovaries, testes were constructed by using Illumina Solexa high-throughput sequencing technology. The four libraries produced a dataset of 40,931,727 raw reads in total: 10,482,821; 10,641,766 reads from the females and males. 9,989,208; 9,817,932 reads from the ovaries and testes (Table 1). These raw reads have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (accession number: GSE80536). After removing junk sequences, simple sequences, sequences longer than 26 nt or shorter than 18 nt and filtering the RFam (rRNA, tRNA, snRNA, snoRNA, and other RfamRNAs), B. dorsalis miRNAs and Repbase sequences, a total of 6,210,904; 7,237,349; 5,445,095 and 6,894,823 for females, males, ovaries and testes mappable clean reads were gained, respectively (Table 1). The length distribution of the total small RNA reads in the data set showed the majority of the small RNAs ranged from 19 nt to 22 nt and a
peak at 21nt, which is the typical length of the mature miRNAs (Fig 1). Each sample’s length distribution showed in S1 Fig.

Identification of known and novel miRNAs from *B. dorsalis*

By filtering the data set and blasting against the known mature miRNAs and miRNA precursors in miRBase 20.0 (http://www.mirbase.org), we identified 141 miRNAs in the females library, 145 miRNAs in the males library, 127 miRNAs in the ovaries library, 143 miRNAs in the testes library and a total of 183 miRNAs in the combined data set (S1 Table). Low-energy, fold-back structures, and 18–24 nt mature miRNAs of miRNA precursors distinguish miRNAs from other small RNAs. The name of the most reads miRNA represented the particular miRNA and other variants. The expression levels of *B. dorsalis* known miRNAs ranging from 404222.33 counts for the most abundant to single count.

In addition to the known miRNAs, the remaining sequences were aligned with the whole genome sequence (WGS) of *B. dorsalis* to identify novel miRNAs. Sequences that did not

---

**Table 1. Distribution of sequenced reads from raw data to cleaned sequences.**

| type                | females | %   | ovaries | %   | males | %   | testes | %   |
|---------------------|---------|-----|---------|-----|-------|-----|--------|-----|
| Total               | 10,482,821 | 100 | 9,989,208 | 100 | 10,641,766 | 100 | 9,817,932 | 100 |
| 3ADT&length filter  | 2,811,145 | 26.82 | 3,686,552 | 36.91 | 1,956,525 | 18.39 | 1,583,814 | 16.13 |
| Junk reads          | 33,056 | 0.32 | 12,664 | 0.13 | 19,497 | 0.18 | 18,282 | 0.19 |
| Rfam                | 1,408,998 | 13.44 | 833,784 | 8.35 | 1,402,723 | 13.18 | 1,298,205 | 13.22 |
| Repeats             | 21,617 | 0.21 | 12,658 | 0.13 | 28,793 | 0.27 | 25,562 | 0.26 |
| rRNA                | 1,139,864 | 10.87 | 734,069 | 7.35 | 1,105,241 | 10.39 | 1,008,233 | 10.27 |
| iRNA                | 119,424 | 1.14 | 35,617 | 0.36 | 118,800 | 1.12 | 127,510 | 1.30 |
| snoRNA              | 9,531 | 0.09 | 13,203 | 0.13 | 7,776 | 0.07 | 8,387 | 0.09 |
| snRNA               | 18,542 | 0.18 | 7,275 | 0.07 | 6,084 | 0.06 | 7,155 | 0.07 |
| other Rfam RNA      | 121,637 | 1.16 | 43,620 | 0.44 | 164,822 | 1.55 | 146,920 | 1.50 |
| Clean reads         | 6,210,904 | 59.25 | 5,445,095 | 54.51 | 7,237,349 | 68.01 | 6,894,823 | 70.23 |

doi:10.1371/journal.pone.0159591.t001
mapped to the conserved sequences in miRBase 20.0 and previously identified known miRNAs of *B. dorsalis* were considered as novel or species-specific miRNAs. Using MIREAP software (http://sourceforge.net/projects/mireap/), 120 new novel miRNAs were obtained in the four libraries altogether (S2 Table). Amongst these novel miRNAs, 74, 74, 112 and 94 were detected in the females, males, ovaries and testes, respectively. Notably, almost all these novel miRNAs are either -5p or -3p arms of proposed precursors, which is coordinated with previous reports [25, 26]. The precursor secondary structure of each novel miRNA was constructed and the negative-folding free energies of their secondary structures range from 63.4 to 15.0 kcal/mol (S2 Table). In addition, secondary structures of one known and one novel miRNAs are shown in Fig 2.

**Figure 2. MiRNA hairpins.** (A) The predicted hairpin structure of the miR-8 hairpin, colored as miRNA and miRNA*. Red reflects miR-8-5p and yellow reflects miR-8-3p. (B) The predicted hairpin structure of the novel candidate hairpin, colored as miRNA and miRNA*. Red reflects PC-5p-15170 and yellow reflects PC-3p-3745.

doi:10.1371/journal.pone.0159591.g002

Characterization of known and novel miRNAs

Base composition can affect the physiochemical and biological properties of miRNA including the secondary structures of miRNAs and the activity of enzymes [27,28]. Analysis of the nucleotide bias in miRNAs have been proved a dominant base for uracil (U) at the first nucleotide [29,30]. In our study, we analyzed the first nucleotide bias in all identified miRNAs. The percentage of the four nucleotides at first nucleotide showed that U was frequently existed (54.48%) at the 5’ end (Fig 3). The nucleotide bias of miRNAs in insects indicated this phenomenon might be involved in the regulation of the target gene.

We identified a total of 303 miRNAs, of which 183 known miRNAs belonged to 91 families. The conservation profile of the identified miRNAs that matched to other species are presented in Fig 4. Those conserved miRNA families partake the same seed regions and a few different nucleotide in the non-seed sequence regions. The seed sequence (bases 2–8 from the 5’ end) plays a key role in miRNA-target recognition for translational inhibition or mRNA cleavage [31]. The seed region ATCACAG in our study comprised 23 unique miRNAs including miR-2a, miR-2b, miR-5, miR-6, miR-11, miR-13a, miR-13b, miR-308 and miR-994 which belong to the same families demonstrate that miRNAs shared the same seed can regulate the gene expression corporately in different biological processes.
Sex-biased expression of miRNAs

To characterize the sex-biased expression patterns in *B. dorsalis*, the miRNAs from females, males, ovaries and testes library were cross-compared. In our study, we identified 18 female-biased and 16 male-biased miRNAs. Of the sex-biased miRNAs, some are pairs derived from the same precursor (like miR-5-5p and miR-5-3p; miR-8-5p and miR-8-3p), and most of the miRNAs prefer to cluster in the genome and express in a consistent sex-biased pattern (Table 2). Intriguingly, the female-biased and male-biased miRNAs are highly expressed in ovary and testes respectively (S3 Table). This suggests that sex-biased miRNAs are mainly involved in the reproductive function. qRT-PCR experiments were used to validate deep sequencing data by measuring the expression profiles of 10 random selected miRNAs. The results showed that miR-6-3p, miR-989-3p, miR-994-5p, miR-308-3p were significantly more highly expressed in the ovaries, miR-8-3p, miR-1-3p, miR-12-5p, miR-274-5p was preferentially expressed in the testes, while miR-995-3p and miR-276a co-expressed in the ovary and testes (Fig 5). The consistency of the miRNA expression and deep sequencing data indicates that all the analysis in our study is reliable.
Target prediction for sex-biased microRNAs

Intending to understand the physiological functions and biological processes of sex-biased miRNAs in sex determination and gonad differentiation, we annotated the miRNAs and miRNA targets by Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Target prediction was performed by integrating miRanda [32] and TargetScan [33] data. Four female-biased and four male biased miRNAs and their related target gene are listed in Table 3. One target gene of the female-biased miR-989-3p is *doublesex (dsx)* which is considered as the key switch gene in the sex determination of tephritid insects. GO annotation and KEGG pathway analysis were performed to identify functional modules regulated by these miRNAs. GO enrichment analyses of the percentage of genes involved in biological process, cellular component, and molecular component are shown in Fig 6. The KEGG pathway analysis revealed 250 pathways that were enriched with miRNA targets (data not shown). Neuroactive ligand-receptor interaction, PPAR signaling pathway, gamma-Hexachlorocyclohexane degradation and D-Glutamine and D-glutamate metabolism ranked among the most enriched pathways. The 10 most-enriched Go categories for the target genes of miRNAs and KEGG pathways enriched in target genes of miRNAs are shown in S4 Table and S5 Table.

Discussion

To explore the differential expressed miRNAs between sexes and gonads, we identified and characterized miRNAs from females, males, ovaries and testes in *B. dorsalis* by Solexa deep sequencing. A total of 183 known and 120 novel miRNAs were gained from the four libraries. The length distribution of the total small RNA reads in data set presented that the dominant size was 21 nt followed by 22 and 20 nt sequences which is the typical length of the mature miRNAs, but a very low in 24, 25, 26 nt. In the previous report, only a total of 123 known and 60 novel miRNAs were identified in *B. dorsalis* [25]. The available of the whole genome sequence (WGS) of *B. dorsalis* enlarge the finding of known and novel miRNAs in our study.

Among these miRNAs in our study, 44 pairs of known miRNAs and miRNA’s (postfixed with 3p and 5p) were observed. The miRNA and miRNA\(^*\) sequences from 44 pairs either share similar or differential expression. miRNA and miRNA\(^*\) are the products of the same pre-miRNA and functioned differently [34]. miRNA is stable and loaded into RISC to recognize the target gene, while miRNA\(^*\) is considered inactive and degraded immediately in cytosol [35,36]. But the recent reports showed that some miRNA\(^*\) with high expression play potential roles in regulation [37]. The 3’ and 5’ arm miR-10 are co-expressed and both target *Hox* genes in Drosophila [38–40]. These miRNA precursors may produce functional molecules from both arms. In fact, miRNA’s could have some endogenous targets; Okamura found that *D. 

Table 2. MicroRNAs with sex-biased expression and genome location.

| female-biased | male-biased: |
|--------------|--------------|
| miR-996-3p  | JFBF010000218.1 | miR-8-5p | JFBF01000093.1 |
| miR-994-5p  | JFBF01000147.19 | miR-999-3p | JFBF01000069.1 |
| miR-318-3p  | JFBF01000147.1 | miR-306-5p | JFBF0100027.1 |
| miR-308-3p  | JFBF01000075.1 | miR-309-5p | JFBF01000054.1 |
| miR-10-5p   | JFBF01000063.1 | miR-10-5p | JFBF01000063.1 |
| miR-309-3p  | JFBF01000054.1 | miR-309-3p | JFBF01000054.1 |
| miR-6-3p    | JFBF01000054.1 | miR-6-3p | JFBF01000054.1 |
| miR-4-3p    | JFBF01000054.1 | miR-4-3p | JFBF01000054.1 |

doi:10.1371/journal.pone.0159591.t002
melanogaster miR-iab-4-3p can involved in endogenous regulatory networks by target abrupt gene [41]. This differential expression of the 3p or 5p miRNA from the both arms of precursors are associated with specific tissue [42], and indicate a clue in miRNA evolution [43].

In our study, we identified 91 families from the 183 known miRNAs. miR-2a, miR-2b, miR-5, miR-6, miR-11, miR-13a, miR-13b, miR-308 and miR-994 with same seed region ATCA-CAG in our study is the most abundant family. miR-2 (miR-2, miR-13a and miR-13b) is involved in the metamorphosis through repressing the Krüppel homolog 1 (Kr-h1) expression in Blattella germanica [44]. While in Drosophila, miR-2 control apoptosis by targeting potential proapoptotic genes (reaper, grim and sickle) [45]. miR-10 members are considered a close interplay in regulation Hox [46]. All these signs show that miRNA family is conserved among

---

**Fig 5. Real-time quantitative PCR validates expressions of 10 known miRNAs.** The amount of expression was normalized to the level of α-tubulin. Error bars show SD from three independent experiments with three triplicates each and asterisks ( * or ** or *** ) indicate significant differences (P< 0.05 or P<0.01 or P<0.001, respectively) compared to the relevant control in a two-tailed t-test.

doi:10.1371/journal.pone.0159591.g005
| miRNA   | miRNA sequences (5’-3’) | Target gene | Gene annotation                                      | TargetScan | miRanda | Total |
|---------|------------------------|-------------|------------------------------------------------------|------------|---------|-------|
| miR-989-3p | UGUGAUGUGACGUAGUGG   | TRPL        | Transient-receptor-potential-like protein            | 1          | 1       | 2     |
|         |                        | CU01        | Cuticle protein                                      | 1          | 1       | 2     |
|         |                        | ACOD        | Acyl-CoA desaturase                                   | 1          | 1       | 2     |
|         |                        | DSX         | doublesex                                            | 1          | 1       | 2     |
| miR-994-5p | UAAGGAAAUAGUAGCCGUGAUU | PP1B        | Serine/threonine protein phosphatase                  | 1          | 1       | 2     |
|         |                        | TRYE        | Trypsin epsilon                                      | 1          | 1       | 2     |
|         |                        | CP6A2       | Cytochrome P450                                       | 1          | 1       | 2     |
| miR-5-3p  | UAUCACAGUGAUUUCUUGU    | SP24D       | Serine protease                                      | 1          | 1       | 2     |
|         |                        | RERGL       | Ras-related and estrogen-regulated growth inhibitor-like protein | 1          | 1       | 2     |
| miR-318-3p | UCACUGGGCUUUGUUAUCUA  | ATPB        | ATP synthase                                         | 1          | 1       | 2     |
|         |                        | TITIN       | Titin                                                | 1          | 1       | 2     |
|         |                        | RRP44       | Exosome complex exonuclease                           | 1          | 1       | 2     |
| miR-8-5p  | CAUCUUACCGGCAGCAUAGA  | REN11       | Renin-1                                              | 1          | 1       | 2     |
|         |                        | PGK         | Phosphoglycerate kinase                               | 1          | 1       | 2     |
|         |                        | URM1        | Ubiquitin-related modifier 1 homolog                 | 1          | 1       | 2     |
|         |                        | HSBP1       | Heat shock factor-binding protein                     | 1          | 1       | 2     |
| miR-252-5p | CUAAGUACUGUGCCGCAGGA  | YTV2        | Uncharacterized zinc metalloprotease                  | 1          | 1       | 2     |
|         |                        | SNMP1       | Sensory neuron membrane protein                      | 1          | 1       | 2     |
|         |                        | LPH         | Lactate-phlorizin hydrolase                          | 1          | 1       | 2     |
| miR-274-5p | UUUGUGAGACACUAACCGGU  | TRY         | Trypsin                                              | 1          | 1       | 2     |
|         |                        | MDR49       | Multidrug resistance protein                         | 1          | 1       | 2     |
|         |                        | NLTP        | Non-specific lipid-transfer protein                   | 1          | 1       | 2     |
| miR-12-5p  | UGAGUAAUACUACGGUACUGU | SER1        | Serine proteases                                     | 1          | 1       | 2     |
|          |                        | MTH11       | G-protein coupled receptor                            | 1          | 1       | 2     |
|          |                        | COQ6        | Ubiquinone biosynthesis monoxygenase                 | 1          | 1       | 2     |

doi:10.1371/journal.pone.0159591.t003
species, but can alter target gene via different seed sequences during the course of evolution [47]. Previous studies showed that the first and ninth nucleotide which limit the seed region at the 5’ end of mature miRNA are enriched with uridine (U) and vital to miRNA-target recognition [31]. In our study, we found that uridine (U) was the most abundant (54.48%) at first nucleotide but not at ninth nucleotide, which is coordinated with previous reports [25].

In *D. melanogaster*, amounts of female-biased and male-biased miRNAs have found and distributed mostly in the gonad [4]. It is reported that these sex-biased miRNAs are encoded within the sex-biased genes and associated with the reproductive function [43,48]. Our study obtained 18 female-biased miRNAs and 16 male-biased miRNAs. Among these miRNAs, miR-989-3p, miR-994-5p, miR-996-3p, miR-92b-3p, miR-306-5p, miR-12-5p were also sex-biased expression in *D. melanogaster* [4], indicating the conservation of these sex-biased miRNAs among different species. miR-5-3p/miR-5-5p, miR-4-3p/miR-6-3p/miR-309-3p, miR-318-3p/miR-994-5p from female-biased miRNAs and miR-8-5p/miR-8-3p, miR-3477-5p/miR-12-5p, miR-252-5p/miR-1000-5p from male-biased miRNAs are clustered in *B. dorsalis* genome, suggesting that those clustered miRNA in *B. dorsalis* is transcribed in multicistronic primary-miRNA. Unexpectedly, all the sex-biased are located in the autosome rather than the male-biased miRNAs emerged in the X chromosome as reported [4]. The X chromosome cluster miR-982-5p, miR-983-5p, miR-984-5p, miR-973-3p, miR-9369-5p, miR-4966-5p, miR-975-5p in our library have a low expression in all samples, which is different from previous reports in *D. melanogaster*, the enriched X chromosome cluster miR-982/983/984 and miR-973/4966/975 are highly expressed in testes of *D. melanogaster* [4]. The divergence of X chromosome cluster miRNAs between *D. melanogaster* and *B. dorsalis* may be the results of a lossment of male genes and male-biased miRNAs from the X chromosome in evolution [4,49].

By using miRanda and TargetScan software, the target genes of sex-biased and gonad-specific miRNAs were predicted. We found one target gene of the female-biased miR-989-3p is *doublesex (dsx)* which is considered as the key switch gene in the sex determination of *Bactrocera* pests. The primary signal of sex-determination cascade in the tephritid insects relies on a Y-linked male-determining factor (M-factor) [50], while *dsx* is the bottom gene of the cascade and conserved in the regulation of sexual differentiation [51,52]. The linkage between miR-989-3p and *dsx* in our study indicate that sex-biased miRNAs may not only associated with the reproductive function but also involved in the sex-determination by regulating the cascade genes in *B. dorsalis*. Fagegaltier et al. (2014) found that *let-7* as a ecdysone-mediated signaling...
factor affect the expression of sex-specific genes including *sexlethal (sxl)*, *dsx* and yolk protein gene (yp1), which to some extent, maintaining sexual identity in the soma of *D. melanogaster*. Also, there are no miRNAs on the Y chromosome in *B. dorsalis*. While in *Bombyx mori*, a W-chromosome specific small RNA (piRNA) have an effect on the sex-specific *dsx* splice variants and determined the primary sex in the WZ sex determination system [53]. The deficiency of Y-chromosome miRNAs in Diptera species indicates a different sRNA-mRNA interplay mechanism in XY system.

In this study, the potential miRNAs in females, males, ovaries and testes of *B. dorsalis* were identified and characterized using deep sequencing, and 183 known miRNAs, 120 novel miRNAs were abtained. The different expression between sexes and gonads suggested that these miRNAs may play important roles in reproduction processes such as spermatogenesis and oogenesis. Further function analyses of the sex-biased miRNAs and their target genes will be of great significance to better understand the somatic and germline sex determination mechanism in *B. dorsalis* and to develop new control approaches by miRNA interruption.

**Materials and Methods**

**Sample preparation and RNA extraction**

*B. dorsalis* were reared on artificial diet and cultured at 28°C under a photoperiod of 12 h light:12 h dark described previously in our laboratory [54]. Newly emerged virgin females and males were separated by sex and after 12 days mature female and male adults were dissected. After dissection, the gonads and the rest adult samples were immediately stored in RNAlater® Solution (Ambion). To identify the small RNAs involved in *B. dorsalis* gonad proliferation and sex differentiation, four small RNA libraries were constructed from the mature adults (without ovaries and testes respectively) and gonads samples. Total RNAs were extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer’s protocol, the quantity and purity were examined using an Bioanalyzer 2100 (Agilent, CA, USA).

**Small RNA library construction and deep sequencing**

Small RNA libraries were generated from the four RNA samples with the TruSeq Small RNA Sample Prep Kits, following manufacturer’s guide (Illumina, San Diego, USA). The 18–30 nt length RNA fractions were separated by gel and ligated sequentially to 3’ and 5’ adaptors. Ligation products were then reverse transcribed using the primer on the 3’ adaptor and 15 PCRs amplified on the first strand synthesis production with both adaptor sequence primers. Ultimately, PCR products were purified, validated and sequenced by LC-Sciences using an Illumina Hiseq2500.

**Small RNA sequence bioinformatic analysis**

After removing low quality reads and adaptor sequences from the raw datas, large amounts of clean small RNAs were analyzed by BLAST against *B. dorsalis* mRNAs database [24], RFAM and Repbase to identify possible mRNA, rRNA, tRNA, snRNA, snoRNA and other ncRNAs. The remaining clean reads were aligned to the miRNA precursors/mature miRNA sequences in miRBase and previously identified miRNAs of *B. dorsalis* [25], with the matched or one mismatch sequences, were identified as known miRNAs. The unmapped sequences after known miRNA identification were aligned to the genome of *B. dorsalis* and the hairpin RNA structures containing sequences were predicated from the flank 80 nt sequences using RNAfold software complying with criteria from the pre-miRNA statistics in miRBase to identify potentially novel miRNAs [55].
Comparison of differential miRNAs

The expression patterns of miRNAs between female and male, female and ovary, male and testes were compared in order to identify sex-biased and gonad specific expressed miRNAs. The abundance of each miRNA in the libraries was transformed through a modified global normalization according to the procedures described before [25]. Each library miRNAs expression were normalized to transcripts per million and those miRNAs which normalized expression were 0 were modified to 0.01. After removing the normalized expression of miRNAs which were less than 1 in four libraries, the normalized data were used to calculate fold-change values \([= \log_2(X/Y)]\) and the log2-ratio plot. The statistically significant difference (threshold of a fold change >2 and \(P\)-value < 0.05) of each library was tested with Fisher test and chi-square test.

miRNA target gene prediction and pathway analysis

According to the procedures described in previous work [55], which provided by LC Sciences Service, we predicted the target genes of highly expressed miRNAs and analyzed the GO terms and KEGG Pathway of these miRNAs. First, TargetScan and MiRanda softwares were applied to identify miRNA binding sites. Then we combined the data which predicted by both softwares and calculated the overlaps. The gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of these highly miRNAs and miRNA targets were annotated [56] and analyzed with the DAVID Bioinformatics Resources Database [57]. The molecular function, cell component, and biological process were used to organize the target genes by functional classification.

qPCR assay of known miRNAs

The expression profiles of ten known miRNAs were investigated in this study. Reverse transcription reactions for mature miRNAs and \(\alpha\)-tubulin as a control were conducted with three biological replicates total RNA (same RNA sources that were used for the sequencing experiments) using miRNA-specific stem-loop primers or an \(\alpha\)-tubulin gene reverse primer. The stem-loop RT primers and gene-specific primers were designed according to previous work [25]. All primers are listed in S6 Table. The reaction system (20 μl) contained 1 μg RNA, 2 pmol stem-loop RT primer or \(\alpha\)-tubulin gene reverse primer, 4 μl 5 xPrimeScript Buffer, 10 nmol deoxynucleotide triphosphates (dNTPs), 20 U Rnase Inhibitor, 100 U PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China) and RNase-free water. The 10 μL reactions consisting of total RNA, stem-loop RT primer or \(\alpha\)-tubulin gene reverse primer, 0.25 mM each of dNTPs and RNase-free water were first incubated for 5 min at 65°C, placed in an ice bath for 2 min, briefly centrifuged, and then other reagents were added. The 20 μL reactions were incubated in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) for 60 min at 42°C for 15 min at 70°C, and then at 4°C for subsequent processes. The reverse transcription products were used for real-time qPCR. qPCRs were performed using SYBR Green qPCR mix following the manufacturer’s instructions in a real-time thermal cycler (Bio-Rad, Hercules, CA, USA). The qRT-PCR program was as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Three biological and technical replicates were performed. Real-time expression data were analyzed by \(2^{-\Delta\Delta C_t}}\) method [58].

Supporting Information
S1 Fig. Length distribution and abundance of small RNAs in the four libraries. (TIF)
S1 Table. Identification of known microRNAs in the four libraries. (XLSX)

S2 Table. Identification of combined novel microRNAs in the four libraries. (XLSX)

S3 Table. Identification of sex-biased microRNAs in the four libraries. (XLSX)

S4 Table. The 10 most-enriched Go categories for the target genes of miRNAs. (DOCX)

S5 Table. The most enriched KEGG pathways for target genes of miRNAs. (DOCX)

S6 Table. Primers used in our study. (XLSX)

Acknowledgments
This research was supported by the earmarked fund for the China Agricultural Research System (No. CARS-27) and Fundamental Research Funds for the Central Universities (No. 2014PY005).

Author Contributions
Conceived and designed the experiments: HZ WP. Performed the experiments: WP JX. Analyzed the data: WP KT HZ. Contributed reagents/materials/analysis tools: HZ. Wrote the paper: WP HZ.

References
1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136(2):215–233. doi: 10.1016/j.cell.2009.01.002 PMID: 19167326
2. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res. 2006; 16(8):995–1004. doi: 10.1101/gr.5217506 PMID: 16825664
3. Fagegaltier D, König A, Gordon A, Lai EC, Gingeras TR, Hannon GJ, et al. A genome-wide survey of sexually dimorphic expression of Drosophila miRNAs identifies the steroid hormone-induced miRNA let-7 as a regulator of sexual identity. Genetics. 2014; 198(2):647–668. doi: 10.1534/genetics.114.169268 PMID: WOS:000343885300026
4. Marco A. Sex-biased expression of microRNAs in Drosophila melanogaster. Open Biol. 2014; 4(4):140024. doi: 10.1098/rsob.140024 PMID: 24694940
5. Marco A, Kozomara A, Hui JH, Emery AM, Rollinson D, Griffiths-Jones S, et al. Sex-biased expression of microRNAs in Schistosoma mansoni. PLoS Negl Trop Dis. 2013; 7(9):e2402. doi: 10.1371/journal.pntd.0002402 PMID: 24069470
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2):281–297. PMID: 14744438
7. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004; 5(7):522–531. doi: 10.1038/ng1379 PMID: 15211354
8. Jiang F, Ye X, Liu X, Fincher L, McKearin D, Liu Q. Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. Genes Dev. 2005; 19(14):1674–1679. doi: 10.1101/gad.1334005 PMID: 15985611
9. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993; 75(5):843–854. PMID: 8252621
10. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993; 75(5):855–862. PMID: 8252622
11. Lai EC, Tomancak P, Williams RW, Rubin GM. Computational identification of Drosophila microRNA genes. Genome Biol. 2003; 4(7):R42. doi: 10.1186/gb-2003-4-7-r42 PMID: 12844358

12. Flynt AS, Lai EC. Biological principles of microRNA-mediated regulation: shared themes amid diversity. Nat Rev Genet. 2008; 9(11):831–842. doi: 10.1038/nrg2455 PMID: 18852696

13. Siomi H, Siomi MC. On the road to reading the RNA-interference code. Nature. 2009; 457(7228):396–404. doi: 10.1038/nature07754 PMID: 19158785

14. Xu P, Vernoo SY, Guo M, Hay BA. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol. 2003; 13(9):790–795. doi: 10.1016/S0960-9822(03)00250-1 PMID: WOS:000182639900030

15. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, et al. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature. 2007; 449(7164):919–922. doi: 10.1038/nature06205 PMID: WOS:000250230600050

16. Caygill EE, Johnston LA. Temporal regulation of metamorphic processes in Drosophila by the let-7 and miR-125 heterochronic microRNAs. Curr Biol. 2008; 18(13):943–950. doi: 10.1016/j.cub.2008.06.020 PMID: 18571409

17. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. MicroRNA expression in zebrafish embryonic development. Science. 2005; 309(5732):310–311. doi: 10.1126/science.1114519 PMID: 15919954

18. Mishima T, Takizawa T, Luo SS, Ishibashi O, Kawahigashi Y, Mizuguchi Y, et al. MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. Reproduction. 2008; 136(6):811–822. doi: 10.1530/Rep-08-0349 PMID: WOS:000262309600015

19. Cutting AD, Bannister SC, Doran TJ, Sinclair AH, Tizard MV, Smith CA. The potential role of microRNAs in regulating gonadal sex differentiation in the chicken embryo. Chromosome Res. 2012; 20(1):201–213. doi: 10.1007/s10577-011-9263-y PMID: 22161018

20. Zhang X, Zheng J, Jagadeeswaran G, Ren R, Sunkar R, Jiang H. Identification and developmental profiling of conserved and novel microRNAs in Manduca sexta. Insect Biochem Mol Biol. 2012; 42(6):381–395. doi: 10.1016/j.ibmb.2012.01.006 PMID: 22406339

21. Ninova M, Ronshaugen M, Griffiths-Jones S. Tribolium castaneum as a model for microRNA evolution, expression and function during short germband development. BioRxiv. 2015:018424.

22. Zhang H, Li H. Photographic guide to key control techniques for citrus disease and insect pests. Beijing: Chinese Agricultural Press; 2012.

23. Shen GM, Dou W, Niu JZ, Jiang HB, Yang WJ, Jia FX, et al. Transcriptome analysis of the oriental fruit fly (Bactrocera dorsalis). PLoS One. 2011; 6(12):e29127. doi: 10.1371/journal.pone.0029127 PMID: 22195006

24. Zheng W, Peng T, He W, Zhang H. High-throughput sequencing to reveal genes involved in reproduction and development in Bactrocera dorsalis (Diptera: Tephritidae). PLoS One. 2012; 7(5):e36463. doi: 10.1371/journal.pone.0036463 PMID: 22570719

25. Huang Y, Dou W, Liu B, Wei D, Liao C, Smagghe G, et al. Deep sequencing of small RNA libraries reveals dynamic expression patterns of microRNAs in multiple developmental stages of Bactrocera dorsalis. Insect Mol Biol. 2014; 23(5):656–667. doi: 10.1111/imb.12111 PMID: 24957108

26. Tariq K, Peng W, Saccone G, Zhang H. Identification, characterization and target gene analysis of testicular microRNAs in the oriental fruit fly Bactrocera dorsalis. Insect Mol Biol. 2016; 25(1):32–43. doi: 10.1111/imb.12196 PMID: 26486729

27. Kudla G, Lipinski L, Caffin F, Helwak A, Zylicz M. High guanine and cytosine content increases mRNA levels in mammalian cells. PLoS Biol. 2006; 4(6):e180. doi: 10.1371/journal.pbio.0040180 PMID: 16700628

28. Shepotinovskaya IV, Uhlenbeck OC. Catalytic Diversity of Extended Hammerhead Ribozymes. Biochem. 2008; 47(27):7034–7042.

29. Dezelj T, Patatnik JF, Huson D, Weigel D. Conservation and divergence of microRNA families in plants. Genome Biol. 2005; 6:P13.

30. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120(1):15–20. doi: 10.1016/j.cell.2004.12.035 PMID: WOS:000226364000006

31. Obad S, dos Santos CO, Petri A, Heidenblad M, Broom O, Ruse C, et al. Silencing of microRNA families by seed-targeting tiny LNA. Nat Genet. 2011; 43(4):371–373. doi: 10.1038/ng.786 PMID: 2142381

32. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res. 2008; 36(suppl 1):D149–D153. doi: 10.1093/nar/gkm995 PMID: 18158296
33. Nam S, Li M, Choi K, Balch C, Kim S, Nephew KP. MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression. Nucleic Acids Res. 2009; 37(suppl 2):W356–W362. doi: 10.1093/nar/gkp294 PMID: 19420067

34. Hutvágner G, McLachlan J, Pasquinelli AE, Bálint É, Tuschi T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. 2001; 293(5531):834–838. doi: 10.1126/science.1062961 PMID: 11452083

35. O’Toole AS, Miller S, Haines N, Zink MC, Serra MJ. Comprehensive thermodynamic analysis of 3’ double-nucleotide overhangs neighboring Watson–Crick terminal base pairs. Nucleic Acids Res. 2006; 34(11):3338–3344. doi: 10.1093/nar/gkj428 PMID: 16820533

36. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008; 9(2):102–114. doi: 10.1038/nrg2290 PMID: 18197166

37. Yang JS, Phillips MD, Betel D, Mu P, Ventura A, Siepel AC, et al. Widespread regulatory activity of vertebrate microRNA* species. RNA. 2011; 17(2):312–326. doi: 10.1261/rna.2537911 PMID: 21177881

38. Stark A, Kheradpour P, Parts L, Brennecke J, Hodges E, Hannon GJ, et al. Systematic discovery and characterization of fly microRNAs using 12 Drosophila genomes. Genome Res. 2007; 17(12):1865–1879. doi: 10.1101/gr.6593807 PMID: WOS:000251359900016

39. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol. 2005; 3(6):e85. doi: 10.1371/journal.pbio.0030085 PMID: 15723116

40. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. Genome Biol. 2004; 5(1):R1–R1. PMID: WOS:000187747000007

41. Okamura K, Phillips MD, Tyler DM, Duan H, Chou Y-t, Lai EC. The regulatory activity of microRNA* species has substantial influence on microRNA and 3’ UTR evolution. Nat Struct Mol Biol. 2008; 15(4):354–363. doi: 10.1038/nsmb.1409 PMID: 18376413

42. Li SC, Liao YL, Ho MR, Tsai KW, Lai CH, Lin WC. miRNA arm selection and isomiR distribution in gastric cancer. BMC genomics. 2012; 13(Suppl 1):S13. doi: 10.1186/1471-2164-13-S1-S13 PMID: 22369582

43. Marco A, Hui HJ, Ronshaugen M, Griffiths-Jones S. Functional shifts in insect microRNA evolution. Genome Biol Evol. 2010; 2(4):353–363. doi:10.1093/nar/gkp294 PMID: 19420067

44. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. Genome Biol. 2004; 5(1):R1–R1. PMID: WOS:000187747000007

45. Stark A, Brennecke J, Russell RB, Cohen SM. Identification of novel Drosophila microRNA targets. PLoS Biol. 2003; 1(3):E60. doi: 10.1371/journal.pbio.0000060 PMID: 14691535

46. Teher D, Heyland-Kroghsbo NM, Lund AH. The miR-10 microRNA precursor family. RNA Biol. 2011; 8(5):728–734. doi: 10.4161/rna.8.5.16324 PMID: 21881411

47. Wheeler BM, Heimberg AM, Moy VN, Sperling EA, Holstein TW, Heber S, et al. The deep evolution of metazoan microRNAs. Evol Dev. 2009; 11(1):3740–3745. doi: 10.1073/pnas.1418522112 PMID: 25775510

48. Fisher B, Weiszmann R, Frise E, Hammonds A, Tomancak P, Beaton A, et al. BDGP insitu homepage. Available: http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl; 2012.

49. Parisi M, Nuttall R, Naiman D, Bouffard G, Malley J, Andrews J, et al. Paucity of genes on the Y chromosome showing male-biased expression. Science. 2003; 299(5607):697–700. doi: 10.1126/science.1079190 PMID: 12511656

50. Willhoeft U, Franz G. Identification of the sex-determining region of the Ceratitis capitata Y chromosome by deletion mapping. Genetics. 1996; 144(2):737–745. PMID: 8889534

51. Hediger M, Burghardt G, Siegenthaler C, Sander C, Marks DS. MicroRNA targets in Drosophila melanogaster and Musca domestica converges at the level of the terminal regulator doublesex. Dev Genes Evol. 2004; 214(1):29–42. doi: 10.1007/s00427-003-0372-2 PMID: WOS:000189303110004

52. Wilkins AS. Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. Bioessays. 1995; 17(1):71–77. PMID: 7702596

53. Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y, et al. A single female-specific piRNA is the primary determiner of sex in the silkworm. Nature. 2014; 509(7502):633–636. doi: 10.1038/nature13315 PMID: WOS:000336457100051

54. Peng W, Zheng W, Handler AM, Zhang H. The role of the transformer gene in sex determination and reproduction in the tephritid fruit fly, Bactrocera dorsalis (Hendel). Genetica. 2015; 143(6):717–727. doi: 10.1007/s10709-015-9869-7 PMID: 26481008
55. Du Y, Wang X, Wang B, Chen W, He R, Zhang L, et al. Deep sequencing analysis of microRNAs in bovine sperm. Mol Reprod Dev. 2014; 81(11):1042–1052. doi: 10.1002/mrd.22426 PMID: 25279827

56. Ashburner M, Bergman CM. Drosophila melanogaster: a case study of a model genomic sequence and its consequences. Genome Res. 2005; 15(12):1661–1667. doi: 10.1101/gr.3726705 PMID: 16339363

57. Sayers EW, Barrett T, Benson DA, Bolton E, Bryant SH, Canese K, et al. Database resources of the national center for biotechnology information. Nucleic Acids Res. 2011; 39(suppl 1):D38–D51. doi: 10.1093/nar/gkq1172 PMID: WOS:000285831700009

58. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001; 25(4):402–408. PMID: 11846609