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Computational Identification of 99 Insect MicroRNAs Using Comparative Genomics*

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Abstract: In recent years, much effort has been made in identifying microRNA (miRNA) genes from mammals, insects, worms, plants, and viruses. Continuing the search for more miRNA genes is still important but difficult. This paper presents a computational strategy based on comparative genomics analysis. The algorithm was used to scan four invertebrate genomes, *Drosophila melangoster*, *Bombyx mori*, *Apis mellifera*, and *Anopheles gambiae*, which are either model organisms or medically/economically important insects. 99 new miRNA genes were predicted from the four insect species which can be grouped into 17 miRNA gene families, of which 10 of the miRNA families are insect-specific. Sequence similarity analysis showed that 16 of the newly predicted insect miRNAs belong to the K-box, GY-box, and Brd-box miRNA families which are important participators in Notch-related pathways. To test the validity of the algorithm, 39 predicted insect miRNA genes from *D. melangoster* and *A. mellifera* were selected for further biological validation. 34 (87%) predicted miRNA genes' transcripts were successfully detected by reverse transcription-polymerase chain reaction experiments. Thus, this strategy can be used to efficiently screen for miRNA genes conserved cross species.

Key words: microRNA (miRNA); insect; comparative genomics; reverse transcription-polymerase chain reaction

Introduction

MicroRNAs (miRNAs) are a class of ~22 nt evolutionary conserved endogenous non-coding RNAs that regulate their target mRNA expressions by either inactivating or degrading mRNA genes. Combinations of computational and biological methods have identified more than 1000 miRNAs in animals, plants, and viruses, including *Homo sapiens*[1-5], *Mus musculus*[1,3,5,6-8], *Rattus norvegicus*[3], *Gallus gallus*[9], *Danio rerio*[2], *Xenopus laevis*[10], *Drosophila melanogaster*[11], *Drosophila pseudoobscura*[11], *Caenorhabditis elegans*[12,13], *Caenorhabditis briggsae*[12,13], *Arabidopsis thaliana*[14-16], *Zea mays*[17], *Oryza sativa*[15,16,18], the *Epstein Barr virus*[19], and the HIV virus[20,21]. It is believed that more miRNA genes exist than are known. At the time this paper was in preparation, there were 462 human miRNA genes deposited in miRbase[22]. However, microarray analyses have suggested that there may be more than 800 miRNA genes in the human genome[23]. Thus, at least half of the human miRNA genes are still unidentified.

There are two strategies for identifying new miRNA genes. One method is to clone ~22 nt short sequences

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by gel purification and direct sequencing and then to identify the putative miRNA precursors with bioinformatics methods. The other method is to scan the entire genome to predict candidate miRNA precursors and to detect mature miRNAs experimentally. Several algorithms have been developed to identify miRNA genes from mammals, insects, worms, and plants. Lim et al.[12] used seven features of 50 known *C. elegans* miRNAs in the miRScan program to conduct a genome-wide scan. In total, 88 *C. elegans* miRNAs can be clustered into 48 gene families. MiRScan was also used to find 188 human candidate miRNAs with scores over 10 (a cutoff for evaluating a putative miRNA)[2]. Lai et al. developed miRseeker to identify 48 new miRNAs in *D. melanogaster*[11]. MIRcheck[15] and MIRFINDER[16] were developed for plants. MiRAlign was designed to align the secondary structures of known pre-miRNAs to detect miRNAs[24]. Most algorithms use sequence and structure alignment information to discover conserved miRNAs as described in the review by Kim and Nam[25]. Recently, some *ab initio* algorithms have been developed to predict miRNA genes, using methods independent of comparative genomics analysis[26,27].

Although the first miRNA, lin-4, was discovered in *C. elegans*, less miRNA genes have been identified in invertebrate than in mammals. This study used a simple computational strategy based on comparative genomics, RNA secondary features analysis, and the miRScan scoring system to detect invertebrate miRNAs. The algorithm was used to scan four insect genomes, *Drosophila melanogaster*, *Bombyx mori*, *Apis mellifera*, and *Anopheles gambiae*, which are either model organisms or medically/economically important insects. In total, 144 pre-miRNAs were predicted, of which 99 of the miRNA genes have not been previously reported. Reverse transcription-polymerase chain reaction (RT-PCR) experiments were performed to validate the predicted miRNA genes from fruit fly and honeybee. Among the 39 predicted miRNAs selected for RT-PCR verification, transcripts of 34 predicted insect miRNAs were successfully detected.

1 Data and Methods

1.1 Data set

1.1.1 Genome data

The genome data of *D. melanogaster*, *A. mellifera*, and *A. gambiae* were downloaded from the NCBI ftp site (ftp://ftp.ncbi.nih.gov/genomes/) and the *B. mori* genome was provided by the Beijing Genomics Institute, Chinese Academy of Sciences. Noncoding regions, including intergenic regions, introns, and UTRs, were extracted from the genbank format files by Perl script.

1.1.2 MiRNA data

MiRNA sequences were retrieved from miRNA Registry 5.0 (ftp://ftp.sanger.ac.uk/pub/databases/Rfam/miRNA/)[22,28]. Version 5.0 contains 11 species with 1345 precursor entries for 1298 miRNAs.

1.1.3 3'-untranslated region (3'-UTR) data

UTR sequences used to predict the miRNAs targets were downloaded from the UTResource database (http://bighost.area.ba.cnr.it/BIG/UTRHome/)[29]. 318 3'-UTR sequences of *B. mori*, 92 of *A. mellifera*, 170 of *A. gambiae*, and 11 585 of *D. melanogaster* were extracted from the database.

1.2 Computational algorithm

1.2.1 Identification of potential mature miRNA segments

The genome of *B. mori*, *A. mellifera*, and *A. gambiae* were aligned with that of *D. melanogaster* by the BLAST program, the 19-35 nt short fragments which conserved between two insect species were remained as potential mature miRNA loci.

1.2.2 Determination of putative miRNA precursors

All the short conserved fragments were used as seeds for extending to obtain potential miRNA precursors. Then, the seeds were stretched to 3'- and 5'-flanking sequences from 60 to 110 nt with steps of 3 nt. The secondary structures of the extended candidate sequences were predicted by the RNAfold program with the default parameters (http://rna.tbi.univie.ac.at/)[30]. Potential miRNA precursors were obtained with three filters: (1) both putative precursors extracted from the two aligned insect genomes can be folded into good hairpin structures, (2) the free energies of the putative precursors predicted by RNAfold are less than – 84 kJ/mol, and (3) the conserved fragments (potential mature miRNAs) are located in the stem region of the hairpin structure[31].

1.2.3 MiRScan scoring system

Finally, all the candidate precursors were scored by miRScan (http://genes.mit.edu/mirscan/index.html). The candidate sequences with scores higher than 10

were regarded as putative miRNA precursors.

1.3 Insect miRNA target gene prediction

MiRNA target genes were predicted by miRanda with a cutoff of 100[32] and targetScan with a cutoff of 5[33].

1.4 RT-PCR validation of predicted miRNA genes

In light of our present understanding of miRNA transcription, upstream and downstream 250 nt flanking sequences of the 35 insect miRNA precursors were extracted from the latest fly and honeybee genome sequences to design the PCR primers. The selected miRNAs contained all the novel fruit fly and honeybee candidates. The other two miRNA clusters were extended up/downstream 250 nt according to the position of the first/last miRNA in the cluster. The primers used to detect the pre-miRNA transcripts are listed in Table S1 at http://bioinfo.au.tsinghua.edu.cn/sup/miRNA_Predict/insect_miRNA_supp.htm. Total RNA was isolated with TRIzol reagent following the manufacturer's protocol (Life Technologies, Gaithersburg, MD, USA). First-strand cDNA was synthesized from total RNA with the SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen) and oligo (dT) as primers. At the same time, a control experiment was designed to verify that the total RNA was not contaminated by genomic DNA. The miRNA transcripts were validated by PCR performed in accordance with standard procedures with 2 μmol/L of each primer and 2 U Taq DNA polymerase (Promega Corporation). The products were resolved by electrophoresis on 1.2% *w*/*v* agarose gel in TAE buffer (40 mmol/L Tris–acetate, 2 mmol/L $Na_2EDTA{\cdot}2H_2O$) and stained by GoldView™ (SBS Genetech). DNA bands were quantified using a UVP-GDS-8000 system UV transilluminator (UVP, Inc).

2 Results

2.1 Predicted insect miRNA genes

The computational strategy flowchart is shown in Fig. 1. In total, 144 putative miRNAs genes were successfully identified, of which 99 were new insect miRNA genes not previously reported. 55 of the predicted miRNA genes have no homology to known miRNA (Fig. 2). 36 pre-miRNAs were discovered from *A. mellifera*, including 13 new miRNAs, 11 homologies to known miRNAs, and 12 miRNAs published in miRBase. 36 pre-miRNAs were discovered from *B. mori*, including 7 new miRNAs and 29 homologies to known miRNAs. 30 pre-miRNAs were discovered from *A. gambiae*, including 16 new miRNAs, 4 homologies to known miRNAs, and 10 miRNAs published in miRBase. 42 pre-miRNAs were discovered from *D. melanogaster*, including 19 new miRNAs and 23 miRNAs published in miRBase. The detailed descriptions and sequences are given at http://bioinfo.au.tsinghua.edu.cn/sup/miRNA_Predict/insect_miRNA_supp.htm.

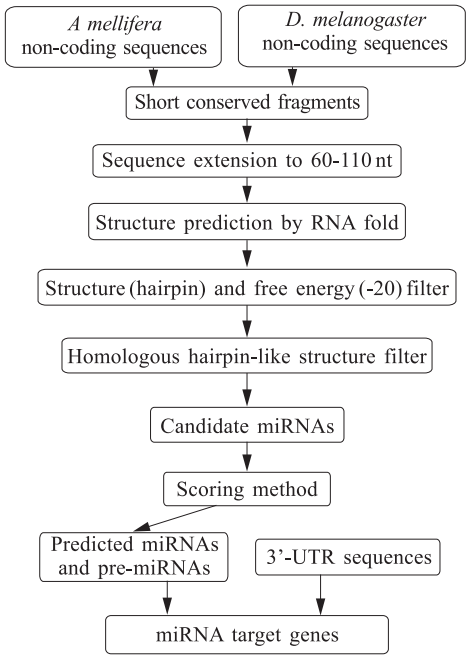

Fig. 1 Flowchart for insect miRNA computational identification procedure

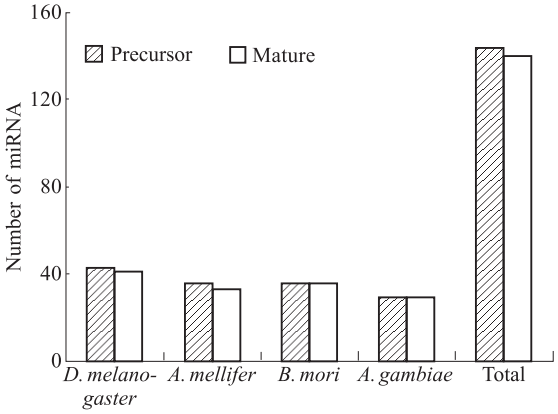

Fig. 2 Statistics for predicted miRNAs in four insect genomes

2.2 Insect miRNA gene families

The conservation of predicted insect miRNAs was then investigated. The sequence similarity of the mature miRNAs showed that 17 miRNA families were conserved in at least three insects. Two insect miRNA families were conserved across all four insect organisms. A homologous analysis with all the known miRNA in miRBase showed that 10 of the insect miRNA families may be insect-specific with no homology in other non-insect species (Table 1). Sequence analysis showed that 10 of the insect miRNAs can be classified into the K-box, GY-box, and Brd-box gene families, which were suggested to play important regulatory roles in the Notch-related pathway[34] (Table 2).

2.3 Insect miRNA clusters

Some of the miRNA genes were found to be clustered adjacent to each other on the genome. Bartel[35] suggested that the miRNA cluster may be from a unique long pri-miRNA transcript, indicating that they have

Table 1 Insect miRNA gene families

MiRNA gene family	*D. melanogaster*	*A. gambiae*	*B. mori*	*A. mellifera*	Other non-insect species
miR-13	+	+	+	+	–
miR-276	+	+	+	+	–
miR-14	+	–	+	+	–
miR-281	+	+	+	–	–
miR-124	+	+	+	–	$+^{15}$
miR-34	+	–	+	+	$+^{14}$
miR-92	+	–	+	+	$+^{17a}$
miR-283	+	–	+	+	–
miR-285	+	+	+	–	–
miR-307	+	+	+	–	–
miR-275	+	+	+	–	–
miR-277	+	+	–	+	–
miR-263	+	+	+	–	–
miR-2	+	–	+	+	cel
miR-133	+	–	+	+	$+^{17b}$
miR-1	+	–	+	+	$+^{9}$
miR-10	+	–	+	+	$+^{13}$

Notes: "+" indicates that miRNA is identified in this paper.

$+^{15}$ indicates that miRNA is conserved in 15 species as (according to miRBase): mmu, hsa, rno, gga, dre, ssc, ggo, age, ppa, ppy, ptr, mml, lla, fru, and tni;

$+^{14}$ indicates that miRNA is conserved in 14 species as (according to miRBase): hsa, mmu, rno, gga, dre, ggo, age, ppa, ppy, ptr, mml, sla, lla, and mne;

$+^{17a}$ indicates that miRNA is conserved in 17 species as (according to miRBase): hsa, mmu, rno, gga, dre, ggo, lca, age, ppa, ppy, ptr, mml, sla, lla, mne, fru, and tni;

$+^{17b}$ indicates that miRNA is conserved in 17 species as (according to miRBase): mmu, hsa, rno, gga, xla, dre, ggo, age, ppa, ppy, ptr, mml, sla, lla, mne, fru, and tni;

$+^{9}$ indicates that miRNA is conserved in 9 species as (according to miRBase): cel, mmu, hsa, cbr, gga, dre, ppa, fru, and tni;

$+^{13}$ indicates that miRNA is conserved in 13 species as (according to miRBase): mmu, hsa, rno, gga, dre, ggo, ppy, sla, age, ppa, mne, fru, and tni.

Full name corresponds to the abbreviation in supplement table at http://bioinfo.au.tsinghua.edu.cn/sup/miRNA_Predict/insect_miRNA_supp.htm.

Table 2 K-box, GY-box, and Brd-box type miRNAs

Motif	miRNA
K-box (cUGUGAUa/AUCACA)	Ame-miR-13a Ame-miR-2a Ame-miR-307 Aga-miR-13b Aga-miR-307 Bmo-miR-s1 Bmo-miR-13 Bmo-miR-2 Bmo-miR-307 Dme-miR-2a Dme-miR-13b Dme-miR-307-3p
GY-box (GUCUUCC/GGAAGAC)	Bmo-miR-7
Brd-box (AGCUUUA/UAAAGCU)	Ame-miR-79 Aga-miR-79 Bmo-miR-9a

similar functions or participate in the same pathway. The potential insect miRNA clusters were analyzed by defining a miRNA cluster as having miRNA genes within 1500 nt of each other. In total, 17 insect miRNAs in *A. mellifera*, *B. mori*, and *D. melanogaster* were located in 7 clusters (Fig. 3). Interestingly, two homologous clusters were found to be conserved between *A. mellifera* and *B. mori*. Another cluster in mosquito was located in the genome repeat region that included Aga-miR-287, Aga-miR-307, Ame-mirR-h15, and Ame-miR-h11. The transcripts, function, and evolutional mechanism of those miRNA clusters need further investigation.

2.4 Experimental verification of predicted insect miRNAs

The efficiency of the computational strategy was tested by biological experiments to validate the predicted miRNA genes. 39 predicted miRNAs genes were selected from *A. mellifera* and *D. melanogaster* for RT-PCR validation. The transcripts of 34 miRNA genes were successfully detected, demonstrating a high accuracy rate of about 87% (34/39) for the algorithm (Table 3 and Fig. 4).

2.5 Insect miRNA target prediction

Two programs, targetScan and miRanda, were used to predict the insect miRNA target genes. These two programs produced very different results with their suggested cutoffs. All the predicted insect miRNA target genes and their annotations are given at http://bioinfo.au.tsinghua.edu.cn/sup/miRNA_Predict/insect_miRNA_supp.htm. This data could be biased since relatively few insect UTR sequences are available at

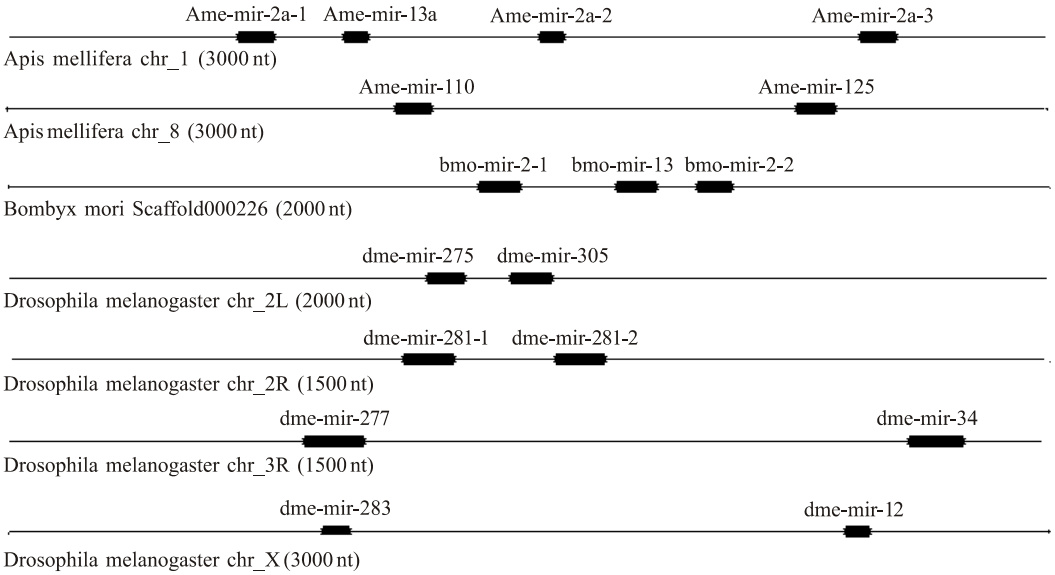

Fig. 3 Insect miRNA clusters

Table 3 Experimental validation of the identified insect pre-miRNA candidates

D. melanogaster		*A. mellifera*		*D. melanogaster*		*A. mellifera*	
mir-f1	+	mir-h1	+	mir-f11	+	mir-h13	+
mir-f2	+	mir-h2	+	mir-f12	+	mir-h15	+
mir-f3	–	mir-h3	+	mir-f13	+	mir-10	+
mir-f4	+	mir-h4	+	mir-f14	+	mir-14	+
mir-f5	+	mir-h5	+	mir-f15	+	mir-34	+
mir-f6	+	mir-210	+	mir-f16	+	mir-283	+
mir-f7	+	mir-h7	–	mir-f17	+	mir-2a-1 & mir-13a	+
mir-f8	+	mir-h8	+	mir-f18	+	mir-100 & mir-125	–
mir-f9	+	mir-h9	+	mir-f19	–		
mir-f10	+	mir-h12	+				

Note: "+" indicates that the expression was validated by RT-PCR.

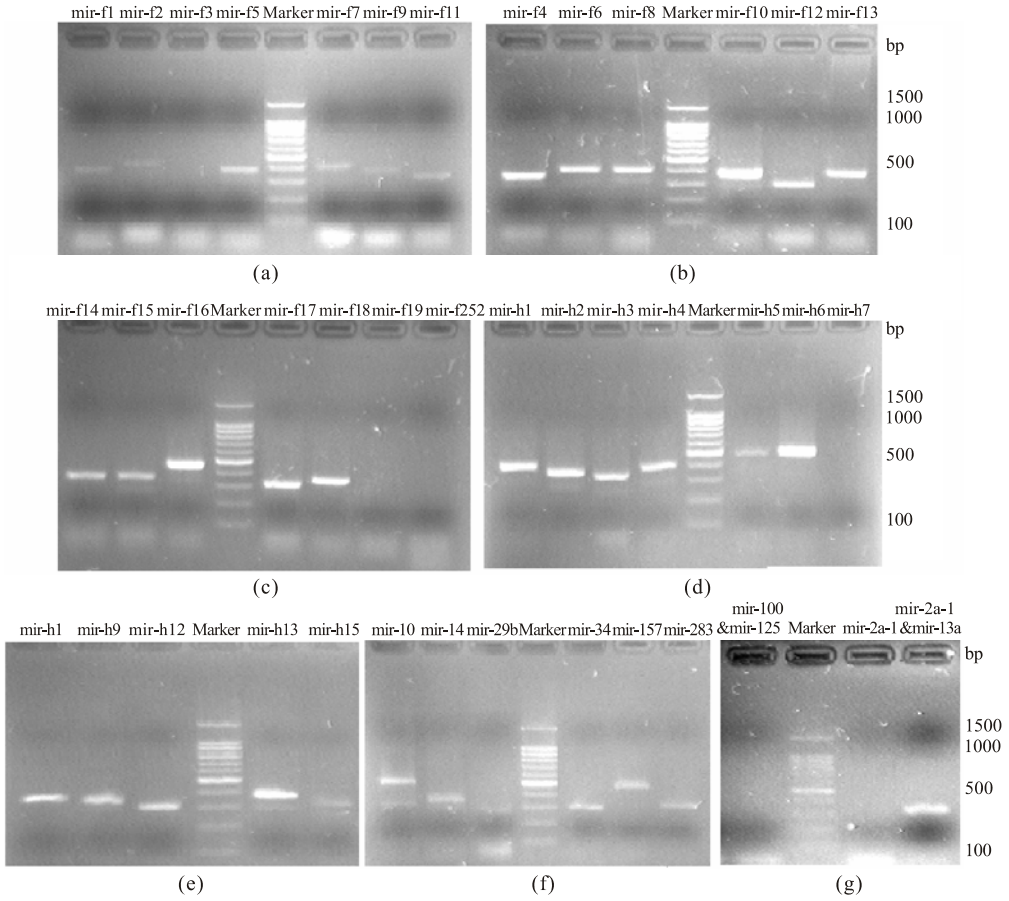

Fig. 4 Experimental validations of the insect pre-miRNAs identified by the computational procedure

the UTResource website and 5'- end of the detected mature miRNAs is quite imprecise. As with previous results in mammals, the potential insect target genes are rich in transcription factors[36].

3 Discussion

Comparative genomic methods have been proven efficient in identifying regulatory elements and non-coding RNA genes. Many computational methods have been developed for miRNA predictions based on comparative genomics, but most can only detect miRNA precursors without annotating mature miRNAs. The present computational strategy identifies mature and precursor miRNAs at the same time. Furthermore, statistical analysis indicates that mature miRNAs are more conserved than precursors; thus, this method that uses the conserved potential mature fragments as seed sequences may uncover more miRNA sequences than other methods that depend on precursor conservation.

10 insect miRNA families were found to have no homologous genes in other non-insect organisms (according to miRBase), indicating they might be insect-specific miRNA genes. Most insects have unique complete or incomplete metamorphosis life cycles[37]. The molecular regulation of larva, pupa, and adult stages of insects that undergo metamorphosis still remains unclear. Since most miRNA genes are closely related to their physical development, further study of these insect-specific miRNA genes may help explain the metamorphosis mechanisms. Preview reports have reported that seven *Drosophila* miRNAs are either up-regulated or down-regulated in conjunction with metamorphosis[38].

Seven miRNA families were conserved across insect, nematode, and vertebrate. Presumably, widely conserved miRNA gene families share a more ancient common ancestor and play an important physiological role. Although the analysis is limited by not knowing the full length of the mRNA genes and their UTR sequences, this primary analysis of miRNA targets genes still reveals some interesting results. For example, *Bmcyc b* predicted as a potential target gene is a

circadian clock gene in *B. mori*. The discovery and validation of circadian clock regulation by miRNA genes in silkworm could be of great interest.

4 Conclusions

Although thousands of miRNA genes have been reported in mammals, insects, worms, plants, and viruses, much effort is still needed to find more potential miRNA genes. This paper reports on 99 new miRNA genes in four insect organisms found using comparative genomics analysis. 55 of the predicted miRNA genes have no homology with any known miRNA in the miRbase database. Thus, this comparative genomics-based strategy is an efficient method for scanning for new miRNA genes, especially in recently reported genomes. RT-PCR experiments successfully verified 34 of the 39 *Drosophila* and *Apis* miRNAs. However, only 45 of the 141 known insect miRNAs were detected by the algorithm. Thus, this computational strategy has a high accuracy rate of about 87% (34/39) but a relatively low sensitivity estimated to be 32% (45/141). Therefore, this comparative strategy tends to predict more reliable miRNA candidate genes with less sensitivity.

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