Isolation of New Micro RNAs from the Diamondback Moth (Lepidoptera: Yponomeutidae) Genome by a Computational Method

Authors: Ellango, R., Asokan, R., Mahmood, Riaz, Ramamurthy, V. V., and Kumar, N. K. Krishna

Source: Florida Entomologist, 97(3) : 877-885

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.097.0345

ISOLATION OF NEW MICRO RNAS FROM THE DIAMONDBACK MOTH (LEPIDOPTERA: YPONOMEUTIDAE) GENOME BY A COMPUTATIONAL METHOD

R. Ellango[1,*], R. Asokan[1,*], Riaz Mahmood[2], V. V. Ramamurthy[3] and N. K. Krishna Kumar[4]
[1]Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Bangalore, India

[2] Department of Biotechnology, Kuvempu University, Shimoga, India

[3]Network Project on Insect Biosystematics, Division of Entomology, Indian Agricultural Research Institute (IARI), New Delhi, India

[4]Deputy Director General (Horticulture), Division of Horticulture, New Delhi, India

*Corresponding author; E-mail: ellango85@gmail.com; asokaniihr@gmail.com

Abstract

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae) is a serious pest of crucifers, worldwide, and has developed high levels of resistance to insecticides including *Bacillus thuringiensis* (Bt). This serious situation requires the identification of non-chemical, alternate, futuristic pest management strategies, which might involve gene silencing. This would first require the identification of small RNAs and their targets, and later their suitable deployment. Micro RNAs (miRNAs) are a class of small RNAs directly involved in various biological functions by regulating gene expression at the post transcriptional level. Finding new miRNAs and predicting their targets from Insect genome would lead to better understanding of their diverse functions. In the present study we have identified 8 new mature miRNAs of *P. xylostella* and predicting their mRNA targets by computational methods. The following 4 miRNAs are reported for the first time in arthropods, viz., miR-244, miR-3529, miR-4704, and miR-7267. We have also analyzed the precursor sequences of the newly identified miRNAs, and we have predicted their secondary structure with minimum free energy index, MFEI, which ranges 0.85 to 1.24. The mRNA target predictions for novel miRNAs were carried out using the Miranda program employing the available putative gene set sequences of *P. xylostella*.

Key Words: biological functions, insect genome, microRNA, Miranda program, *Plutella xylostella*, target prediction

Resumen

La polilla de la col, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae) es una plaga seria de las crucíferas, en todo el mundo, y ha desarrollado un alto nivel de resistencia a los insecticidas que incluyen *Bacillus thuringiensis* (Bt). Esta situación seria requiere de la identificación de estrategias futuristicas, no químicas, alternas de manejo de plagas, lo que podría implicar el silenciamiento de genes. Esto requeriría primero la identificación de pequeños ARNs y sus posibles objetivos, y luego su despliegue adecuado. Los micro ARNs (miARNs) son una clase de pequeños ARNs directamente implicados en diversas funciones biológicas mediante la regulación de la expresión del gen a nivel post transcripcional. Al encontrar las nuevas miARNs y sus objetivos del genoma de insectos llevaría a una mejor comprensión de sus diversas funciones. En el presente estudio se han identificado 8 nuevos miARNs de *P. xylostella* y sus posibles objetivos por métodos computacionales. Los siguientes 4 miRNAs se reportan por primera vez en los artrópodos: miR-244, miR-3529, miR-4704 y miR- 7267. Hemos analizado también las secuencias precursoras de los miARNs recientemente identificados, y a la vez predicho su estructura secundaria con mínimo de energía libre, MEL, que es desde 0.85 hasta 1.24. Las predicciones objetivo de ARNm para nuevos miRNAs se

llevaron a cabo mediante el programa Miranda empleando las secuencias de genes putativos disponibles de P. xylostella.

Palabras Clave: funciones biológicas, genoma del insecto, microARN, programa de Miranda, *Plutella xylostella*, predicción de objectivo

Plutella xylostella (L.) (Lepidoptera: Yponomeutidae) is the most destructive pest of crucifers such as cabbage, cauliflower worldwide. Managing *P. xylostella* is challenging as it has developed high levels of resistance to numerous and diverse insecticides and field levels of resistance to *Bacillus thuringiensis* (Bt) toxin (Baxter et al. 2005). The physiology, insecticide resistance and insect-plant interactions of the diamondback moth (DBM) have been studied extensively (Vuorinen et al. 2004; Tabashnik et al. 2011; Xie et al. 2012) and there is a dire need to create novel insecticidal agents to cope with this pest. An alternate and effective approach might be to develop the use of gene silencing technology against this pest, which might lead to the development of a variety of novel, environmentally superior approaches to insect pest management. In this regards the potential of utilization of miRNA mimics and inhibitors targeting specific insect genes were first described by Jayachandran et al. (2013).

Knowledge of regulatory non-coding small RNA has emerged over the past decade, particularly MicroRNA (miRNAs) 21-24 nt short RNA molecules, which regulate the expression of messenger RNA by translational repression or degradation. The first, miRNA was described in *Caenorhabditis elegans* (lin-4) and subsequently thousands of such miRNAs and their targets have been discovered in the genomes of plant and animal species and their viruses (Bartel 2004). and there is another class of regulatory small RNA called siRNAs, which has a function similar to that of miRNA, but which differ in their biogenesis (Carmell & Hannon 2004). MicroRNA biogenesis begins with the transcription of long primary miRNA (Pri-miRNA) by RNA polymerase II from the genes that encode non-coding transcripts from introns or even coding regions. The primary transcript is further processed into a short hairpin (stem-loop structure) of around 60-80 nt RNA molecules known as the precursor miRNA (pre-miRNA) by the action of Drosha (Class 2 RNase type III enzyme), in association with Pasha (equivalent to DGCR8 in mammals) (Bartel 2009). The nuclear membrane protein called Exportin-5, facilitates the entry of pre-miRNA into the cytoplasm in presence of Ran-GTP as co-factor. Where the Dicer-1 enzyme removes the terminal loop and produces mature miRNA with 2 nucleotide overhangs on the 3' ends (Hutvágner et al. 2001). Then the mature miRNA are integrated into the RNA inducing silencing complex (RISC) in which Argonaute-1 (Ago-1) proteins play a main stabilizing role during target mRNA binding (Winter & Diederichs 2011). Finally miRNA along with RISC complex targets a complementary messenger RNA (mRNA) for degradation or translation repression, depending on the complementarity between the miRNA and the targets (Pillai et al. 2007).

In insects, miRNA profiles and their role in diverse functions such as development and host-pathogen interactions were studied over the years in selected species because of the lack of complete genome sequences of several insect species (Aravin et al. 2003; Yu et al. 2008). The role of miRNAs in insect development has attracted the most attention mainly because of their conserved functions, which have been proven through several studies on stage-specific or tissue-specific expression of miRNAs during insect development in *Drosophila melanogaster* Meigen (Drosophilidae) (Aravin et al. 2003), *Bombyx mori* (L.) (Bombycidae) (Yu et al. 2008), *Apis mellifera* L. (Apidae) (Behura & Whitfield 2010), and *Aedes aegypti* L. (Culicidae) (Behura et al. 2011). Recently 2 sets of genomic sequences of *P. xylostella* were released in the public domain (You et al. 2013; Jouraku et al. 2013); which included KONAGAbase, a genomic and transcriptome database available online with analytical tools.

A computational method of identifying new miRNAs and predicting their gene targets in available genome sequences of insect species is an efficient tool (Singh & Nagaraju 2008; Zhou et al. 2009). Presently, the insect species with greatest number of miRNAs represented in miRBase is *Bombxy mori* with above 500 mature miRNAs. Finding new miRNA genes remains as an important task for identifying conservative and non-conservative novel miRNAs. This study was carried out to isolate new miRNAs from the *P. xylostella* genome and to predict their gene targets by bioinformatics.

Materials and Methods

Genomic Resource and Prediction of miRNAs

Plutella xylostella genome sequences were obtained from 2 different sources, one on KONAGAbase, a DBM database, which is available online at http://dbm.dna.affrc.go.jp/px/. From this site around 88,530 whole genome shot-

gun (WGS) contig sequences (BAGR-01000001 to BAGR01088530) were downloaded. Another set of whole genome shotgun (WGS) contig sequences (38,756) was retrieved from NCBI, GenBank database (AHIO01000001 to AHIO01038756). In total of 127,286 WGS sequences were first subjected to sorting out the redundant or overlapping sequences, and then the remaining sequences were used for further analysis. A total of 18,020 precursor sequences and 22,462 mature sequences belonging to 104 animal species were obtained from miRBase *v*20 (release in June 2013) at http://www.miRbase.org/ (Kozomara 2011).

A BLASTn search of all the animal miRNA sequences (miRBase *v*20) against the whole genome sequence of *P. xylostella* was carried out in 'BioEdit' version 7.2.2 (Hall 1995) with an E-value ≤ 0.01 and other parameters kept as default. The 2 major criteria used for screening the BLAST results were (1) more than 80% sequence identity between each predicted miRNA and the reference miRNA (known miRNA homologous); and (2) number of mismatches should not exceed more than 3 (Singh & Nagaraju 2008).

Secondary Structure Prediction

From the miRNA predicted genome sequence, we extracted the precursor sequence with flanking ~80 nt upstream and downstream from the mature miRNA region. All the selected pre-miRNA sequences were then submitted to 'Mfold' (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) online software used to predict possible secondary structure (hairpin) and calculate the free energy (ΔG) (Zuker 2003). The pre-miRNA secondary structure should pass all of the following criteria: (1) the free energy of the structure is less than or equal to -18 Kcal/mole; (2) mature miRNA hit should be on the stem region; (3) the A+U content of pre-miRNA sequence should be in the range of 30-70%; and (4) at the mature miRNA region the unpairing (bulge size) should not be more than 7 bp (Singh & Nagaraju 2008; Ghosh et al. 2007).

Further these hairpin candidates were screened for distinguishing real miRNA precursors from pseudo miRNA precursors with similar stem-loops using a random forest prediction model by web-based software MiPred (Jiang et al. 2007). The MiPred also provide details regarding the minimal folding free energy (MFE) of the stem loop structure and the *P* value of randomization test for our newly identified miRNAs. The adjusted minimal folding free energy (AMFE) was calculated by (MFE/length of RNA sequence) × 100 and the minimal folding free energy index (MFEI) was calculated by AMFE/ (G+C)% of RNA sequence (Zhang et al. 2006). Finally the newly identified *P. xylostella* miRNAs were named according to the convention criteria set by Griffith-Jones et al. (2006).

Phylogenetic Analysis

After classifying the newly identified miRNAs into different miRNA families using Rfam web-based server (Griffith-Jones 2004), phylogenetic analyses were performed for the selected miRNA families to understand the evolutionary relationships among them by using MEGA *v*5.0 software (Tamura et al. 2011). The phylogenetic tree was drawn using the UPGMA method with 1000 bootstrap replicates.

Target Prediction

Target prediction for animal miRNAs is complex because of imperfect complementarity between miRNA and their mRNA targets. Initially we performed a local BLAST search against the *P. xylostella* putative gene set (32,800 sequences) at KONAGAbase. The target candidates were then analyzed with target prediction software 'MiRanda' version 3.3a (http:\www.microrna.org/) (Enright et al. 2003) and in accordance with the parameters described by Singh & Najaraju (2008). The 3 major criteria for screening the miRNA-target alignment are as follows: (1) no mismatch at the seed region (positions 2 to 7 count starting from the 5' end of miRNA); (2) not more than 1 G:U paring at the seed region; and (3) not more than 1 gap in the miRNA:mRNA duplex.

Results and Discussion

Identification of new miRNAs provides a significant enhancement of the variety of insect miRNAs and offers perceptions into miRNA evolution, biogenesis and expression in insects (Liu et al. 2010). Earlier, Etebari et al. (2013) compared the miRNA profiles in 2 different *P. xylostella* larval libraries (unparasitized control & parasitized by *Diadegma semiclausum* Hellen; Ichneumonidae). and recently Liang et al. (2013) also constructed a miRNA profile from mixed life stages of *P. xylostella* through transcript sequencing reads aligned against reported arthropod miRNA sequences in miRBase, *v*18. Until now the numbers of miRNAs identified in *P. xylostella* are less than in other insects such as *Bombyx mori* for which 567 mature miRNAs have been deposited in miRBase *v*20. Thus there is a major need to obtain new miRNA information in order to extend the miRNA profile in *P. xylostella*. From this study, we identified 8 new mature miRNAs from genome sequences and predicted their regulatory targets using various online resources and bioinformatics tool (Table 1).

TABLE 1. MATURE MICRO-RNAS NEWLY IDENTIFIED FROM *PLUTELLA XYLOSTELLA* GENOME SEQUENCES. BASES THAT DO NOT MATCH THEIR HOMOLOG ARE DEPICTED BELOW WITH AN ENLARGED FONT SIZE.

S. no	miR ID	Mature miRNA Sequence (5' à 3')	Homolog hit	bp match	Seq Px-ID	Position	Strand
1	Pxy-miR-9b-5p	GCUUUGGUAAUCUAGCUUUAUGA	bmo-miR-9b-5p	23/23	BAGR01036584	1307-1285	-
2	Pxy-miR-9b-3p	A**U**GGAGCUAAAUCGCCAAAGCG	bmo-miR-9b-3p	21/22	BAGR01036585	1268-1247	-
3	Pxy-miR-244-5p	**GUG**UUGGUUGUACAAAGUGGUA**U**U	*crm-miR-244-5p	20/24	AHIO01033500	2096-2115	+
4	Pxy-miR-3389-5p	UCGUAGCCGAUGUUCCACAG**G**AG	bmo-miR-3389-5p	22/23	AHIO01026754	1666-1688	+
5	Pxy-miR-3529-3p	AACAACAAAAUCACUAGUCUUCCA	*hsa-miR-3529-3p	24/24	AHIO01011704	10013-9990	-
6	Pxy-miR-3767	UACACAUUAUUUACUACUA**U**U	ame-miR-3767	20/21	BAGR01038077	1004-1024	+
7	Pxy-miR-4704	**C**ACACUAGGCAUGUGAGUGA**G**U	*hsa-miR-4704-5p	20/22	AHIO01005152	38279-38258	-
8	Pxy-miR-7267	U**ACC**UUGGGUUAGAUUGGAUUGG	*mdo-miR-7267-5p	20/23	AHIO01026583	45809-45827	+

Note: *crm - *Caenorhabditis remanei* (Nematoda); *hsa – Homo sapiens* (Chordata); mdo - *Monodelphis domestica* (Chordata).

Predicting New miRNAs

In order to identify both conserved and non-conserved miRNAs, we used the complete set of Animal miRNA sequencse from miRBase, *v*20 (updated with more miRNAs) aligned against available *P. xylostella* genome sequences. Prediction resulted in finding of 375 mature miRNA hits in the genome sequence. After removal of earlier reported *P. xylostella* miRNAs (Etebari et al. 2013; Liang et al. 2013) in the list, the non-redundant miRNA hits were extracted for further analysis. Usually each animal miRNA has a small precursor length around a 100 bp region that is folded into a stem-loop structure; and this is used to identify the putative Pre-miRNA sequences. The selected mature miRNAs (29 nos) found in the *P. xylostella* genome sequences were scanned to extract ~80 nt upstream and downstream from the miRNA hit. The extracted sequences were considered as precursor sequences of miRNA and used for structural analysis.

Structure Prediction and Validation

The Mfold web server was largely used for the prediction of MicroRNA secondary structure (RNA folding) by energy minimization using nearest neighbor energy parameters (Lewis et al. 2003). Thus from the extracted genome sequences only 65% of pre-miRNAs met the secondary structure predictions with the above listed criteria's by Mfold. The screening resulted in 7 stem loop structures at 63 nt to 75 nt in length and free energy (ΔG) ranges from -22.10 to -37.60 Kcal/mole (Fig. 1). The resulting set of sequences and their respective RNA structures were further analyzed to distinguish true miRNA precursors from the other RNAs with somewhat similar structures. Validating these pre-miRNA sequences by the MiPred online program resulted in the identification of 5 pseduo miRNA precursors and (data not shown). Finally 8 mature miRNAs in 7 precursor sequences with their valid secondary structures were obtained and listed in Table 2.

Phylogenetic Analysis

Insect miRNAs are phylogenetically deeply conserved and are involved in conserved pathways between vertebrates and invertebrates (Asgari 2013). Out of 8 newly predicted miRNAs, the miR-244-5p, miR-3529-3p, miR-4704, and miR-7267 are reported for the first time from the Hexapoda. However these 4 miRNAs homologs are found in various animal phyla (Table 1), and may be considered as novel candidates. All 8 predicted miRNAs were classified into different microRNA families based on their precursor sequences in Rfam web server (Griffiths et al. 2005). This resulted in 2 known families including only 2 miRNAs and

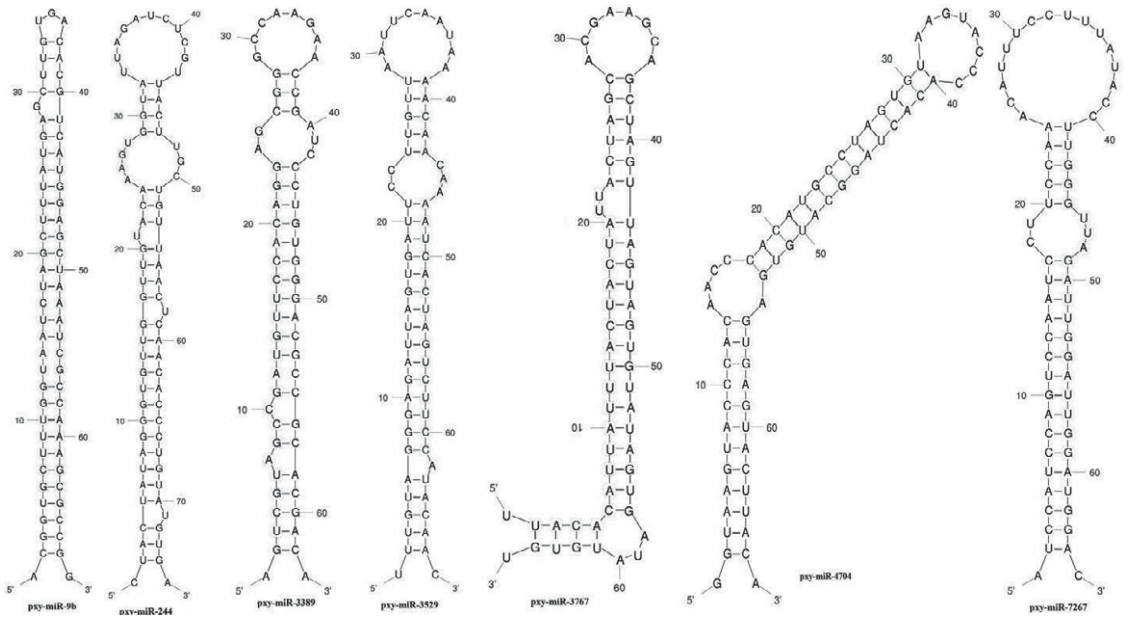

Fig. 1. Secondary structures of newly identified miRNAs from *P. xylostella* (the mature miRNA sequences are highlighted).

the remaining are unknown (Table 3). From these 2 families, we selected the miR-9b family, which we aligned along with our newly identified *P. xylostella* pre-miRNAs for phylogenetic analysis. A phylogenetic tree was constructed for the miR-9b miRNAs based on the Minimum Evolution (ME) method. This tree reveals that the sequence was conserved among the various insect orders. The *P. xylostella miRNA*-9b micro RNAs were grouped along with that of the lepidopteran, *B. mori,* and with a sequence similarity around 80.9% (Fig. 2). On the other hand the dipterans made a sepa-

TABLE 2. PRECURSOR SEQUENCES OF NEWLY IDENTIFIED MIRNAS IN THE *PLUTELLA XYLOSTELLA* GENOME.

S. no	miR ID	Premature Sequence (5' à 3')	bp	MFE	MFEI
1	Pxy-miR-9b-5p & 3p	ACGGTGCTTTGGTAATCTAGCTTTATGAGCTT-GTGACACGTCATGGAGCTAAATCGCCAAAGC-GCCGG	68	-36.0	1.06
2	Pxy-miR-244-5p	CTACTATAGGGTGTTGGTTGTACAAAGTGG-TATTAGATCTCGTTACTTGCTGTTAACTCAA-CACCCTGTATGTGA	75	-26.7	0.89
3	Pxy-miR-3389-5p	AGTCGTAGCCGATGTTCCACAGGAGCGGGC-CAAGAACCGATCCCTGTGGGACGCCGCACGA-CA	63	-33.90	0.85
4	Pxy-miR-3529-3p	TTTGTAGGGAGATTAGTGATTCCTTGTTA-ATTCAATAAAACAACAAAATCACTAGTCTTC-CATACAAC	68	-26.10	1.24
5	Pxy-miR-3767	TTACACATTATTTACTACTATTACTAGCAC-GAAGCAGCTAGTTAGTAGTGTATAGTGATAT-GTGT	65	-22.10	1.05
6	Pxy-miR-4704	GGTAAGTACCCACAACCCACATGCCTAGTGTA-AGTACCCACACTAGGCATGTGAGTGAGTACT-TACA	67	-37.60	1.18
7	Pxy-miR-7267	A T C C A T C C A G T C C A A T C C T T C C A A A-CATTTCCTTTATACCTTGGGTTAGATTGGATTG-GATGGAC	65	-30.19	1.12

TABLE 3. NEWLY IDENTIFIED PRE-MIRNAS FROM THE *PLUTELLA XYLOSTELLA* GENOME ARRANGED INTO RFAM FAMILIES.

S. no	Known Rfam Family	Newly Predicted Pre-miRNAs
1	miR-7	pxy-miR-3529
2	miR-9	pxy-miR-9b

rate clade from the rest of the insect orders, which shows the highly conserved evolutionary relationships among members of the *miRNA*-9b family.

Targets Prediction

We identified the miRNA regulatory target genes in order to pursue functional analyses of miRNAs in regulating various biological processes. MicroRNA regulates the level of transcripts of the target gene at different times and locations (tissues or organs) during development, immunity, or host-microorganisms interaction (Aravin et al. 2003; Xu et al. 2004; Skalsky et al. 2010). Mostly animal amicroRNA will control its target through interaction with the target's 3' untranslated region (UTR) and it may also bind to the coding region and 5' UTR (Bartel 2009; Rigoutsos 2009). Therefore, we used the full lengths of the *P. xylostella* mRNAs (32,800 putative genes) collected from the KONAGAbase for miRNA target prediction. Predicting the targets of animal miRNA can be difficult because of imperfect complementarity between microRNA and target mRNA sequences. The miRNA seed region (2-7 nt from the 5' end) determines both the specificity and stability of the miRNA: mRNA duplex (Lewis et al. 2003). For predicting the miRNA targets, we employed the Miranda Program that helps to optimize sequence complementarity based on position-specific rules and interspecies conservation. All the predicted new miRNAs were aligned against the *P. xylostella* gene set along with the parameters set by Singh & Nagaraju (2008). The resulting miRNA-predicted targets were screened using 3 important filters: (i) no mismatch at seed region, (ii) not more than one G:U paring at the seed region, and (iii) not more than one gap in the alignment. The microRNA may regulate one or several targets and sometimes one miRNA has multiple target sites on the same or on different mRNAs (Brodersen & Voinnet 2009; Shin et al. 2010). Many of the predicted novel miRNAs revealed target multiplicity except that miR-723 had only 1 target (PXGS_V2_024247-GD11426-similar to RING finger protein nhl-1). The miR-3529 and miR-4704 each had 3 targets; miR-7267 had 6 targets; and miR-244 notably had 19 targets and showed high multiplicity over the other 3 novel miRNA candidates (Table 4). All these successfully predicted miRNA targets through the computational method will be accepted with high confidence only after in vivo validation (Pasquinelli 2012). These miRNA target genes involve various functions in metabolism, signaling and transcription.

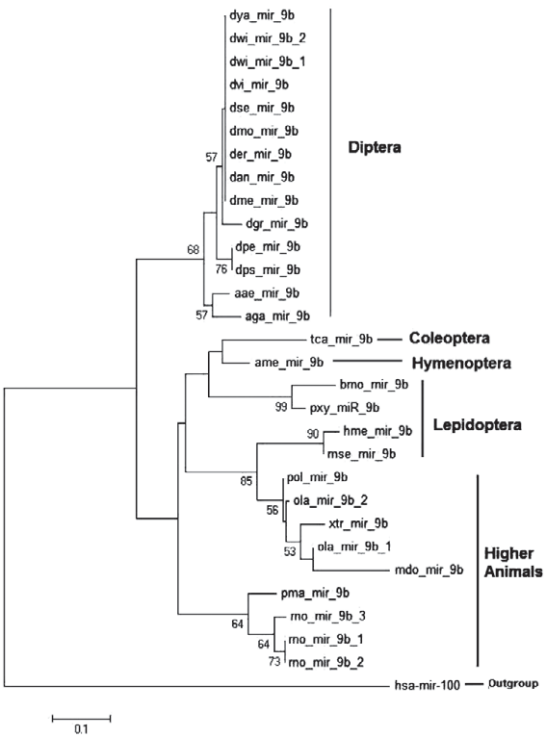

Fig. 2. Phylogenetic tree of miR-9b miRNA families constructed based on the UPGMA method using MEGA v5.0. (miRNAs labels: aae - *Aedes aegypti*; aga – *Anopheles gambiae*; ame – *Apis mellifera*; api – *Acyrthosiphon pisum*; bmo – *Bombyx mori*; dan – *Drosophila ananassae*; der – *D. erecta*; dgr – *D. grimshawi*; dme – *D. melanogaster*; dmo – *D. mojavensis*; dpe – *D. persimilis*; dps – *D. pseudoobscura*; dse – *D. sechellia*; dvi – *D. virilis*; dwi – *D. willistoni*; dya- *D. yakuba*; hme- *Heliconius melpomene*; mse- *Manduca sexta*; tca- *Tribolium castaneum* and pma- *Petromyzon marinus*).

Since the discovery of this small regulator, computational methods serve as an indispensable tool in miRNA gene finding, miRNA target prediction and regulation of miRNA genes. In this study, we identified 8 new miRNAs of *P. xylostella;* among which are 4 miRNAs reported first time in insect species; and their gene targets were predicted using bioinformatics tools. Further investigations are needed to expand our understanding of functional aspects of miRNA regulation in *P. xylostella*. The discovery of miRNAs in the insect genome also may provide a basis for devising novel genetic strategies to manage serious agricultural pests.

TABLE 4. NOVEL MIRNAS AND THEIR GENE TARGETS IN THE *PLUTELLA XYLOSTELLA,* AS PREDICTED BY THE COMPUTATIONAL METHOD. THESE TARGETS AWAIT IN VIVO VALIDATION.

S .no	miRNA ID	KONAGAbase gene ID	Target gene	Position (bp)
1	pxy-miR-244	PXGS_V2_001626	chromatin regulatory protein sir2	117 – 139
		PXGS_V2_004308	hypothetical protein SINV_07222	246 – 268
		PXGS_V2_005113	microtubule binding protein	5620 – 5645
		PXGS_V2_005683	GI22074	3270 – 3294
		PXGS_V2_005865	carbohydrate sulfotransferase 11-like	233 – 256
		PXGS_V2_010462	elongation factor 1 delta	326 – 350
		PXGS_V2_010862	hypothetical protein TcasGA2_TC005652	2632 – 2653
		PXGS_V2_010954	hypothetical protein AaeL_AAEL011101	1144 – 1163
		PXGS_V2_011099	pentatricopeptide repeat-containing protein 2-like	515 – 539
		PXGS_V2_011329	Titin-like protein	1596 – 1619
		PXGS_V2_012072	rCG37751	1557 – 1577
		PXGS_V2_013475	unknown	300 – 323
		PXGS_V2_013837	soluble guanylyl cyclae beta-1 subunit	469 – 494
		PXGS_V2_015675	hypothetical protein TcasGA2_TC016129	807 – 830
		PXGS_V2_018264	CG5112 CG5112-PA	454 – 477
		PXGS_V2_019470	mediator complex, subunit, putative	529 – 544
		PXGS_V2_019475	hypothetical protein LOC100743667	2475 – 2495
		PXGS_V2_021127	BTB/POZ domain-containing protein 2	1 – 25
		PXGS_V2_031463	PR domain zinc finger protein 5 isoform 3	449 – 473
2	pxy-miR-3529	PXGS_V2_012196	unknown	271 – 296
		PXGS_V2_015896	uncharacterized protein C11orf46 homolog	433 – 456
		PXGS_V2_025579	toll	1441 – 1465
3	pxy-miR-4704	PXGS_V2_029496	hypothetical protein AaeL_AAEL012460	372 – 393
		PXGS_V2_019237	UPF0760 protein C2orf29-like	431 – 453
		PXGS_V2_018629	putative fatbody protein 3Rev-G1	23 – 41
4	pxy-miR-7267	PXGS_V2_024083	hypothetical protein LOC100741636 isoform 2	1930 – 1953
		PXGS_V2_017774	TFIIH basal transcription factor complex p52 subunit	70 – 94
		PXGS_V2_011236	caboxypeptidase 4	1444 – 1466
		PXGS_V2_005041	ribophorin	372 – 394
		PXGS_V2_001831	chitin binding protein	1012 – 1034

Acknowledgments

We gratefully acknowledge the financial support received from the Indian Council of Agricultural Research (ICAR), New Delhi through the XIth Plan Network Project on Insect Biosystematics (NPIB). Our sincere thanks are expressed to the Director, Indian Institute of Horticultural Research, Bengaluru, India, for providing the facilities and encouragement.

References Cited

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D. S., Gaasterland, T., Meyer, J., and Tuschl, T. 2003. The small RNA profile during *Drosophila melanogater* development. Insect Biochem. Mol. Biol. 37: 3-9.

Asgari, S. 2013. MicroRNA functions in insects. Insect Biochem. Mol. Biol. 43: 388-397.

Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. Cell 23: 215-233.

Bartel, D. P. 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297.

Baxter, S. W., Zhao, J. Z., Gahan, L. J., Shelton, A. M., Tabashnik, B. E., and Heckel, D. G. 2005. Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. Insect Mol. Biol. 14(3): 327-334.

Behura, S. K., Haugen, M., Flannery, E., Sarro, J., Tessier, C. R., Severson, D. W., and Duman-Scheel, M. 2011. Comparative genomic analysis of *Drosophila melanogaster* and vector mosquito developmental genes. PLoS One 6:e21504.

Behura, S. K., and Whitfield, C. W. 2010. Correlated expression patterns of microRNA genes with age-dependent behavioural changes in honeybee. Insect Mol. Biol. 19: 431-439.

Brodersen, P., and Voinnet, O. 2009. Revisiting the principles of microRNA target recognition and mode of action. Nature Rev. Mol. Cell Biol. 10: 141-148.

Carmell, M. A., and Hannon G. J. 2004. RNase III enzymes and the initiation of gene silencing. Nat. Struct. Mol. Biol. 11: 214-218

Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., and Marks, D. S. 2003. MicroRNA targets in *Drosophila*. Genome Biol. 5: R1

Etebari, K., Hussain, M., and Asgari, S. 2013. Identification of microRNAs from *Plutella xylostella* larvae associated with parasitization by *Diadegma semiclausum*. Insect Biochem. Mol. Biol. 43(4):309-318

Ghosh, Z., Chakrabarti, J., and Mallick, B. 2007. miRNomics-the bioinformatics of microRNA genes. Biochem. Biophys. Res. Commun. 363: 6-11.

Griffiths-Jones, S., Grocock, R. J., Van Dongen, S., Bateman, A., and Enright, A. J. 2006. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 34: D140-D144.

Griffiths-Jones, S., Moxon, S., Marshall, M., Khanna, A., Eddy, S. R., and Bateman, A. 2005. Rfam: Annotating non-coding RNAs in complete genomes. Nucleic Acids Res. 33: D121-D124.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95-98.

Hutvágner, G., Mclachlan, J., Pasquinelli, A. E., Bálint, E., Tuschl, T., and Zamore, P. D. 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293(5531): 834-838.

Jayachandran, B., Hussain, M., and Asgari, S. 2013. An insect trypsin-like serine protease as a target of miRNA: utilization of miRNA mimics and inhibitors by oral feeding. Insect Biochem. Mol. Biol. 43(4): 398-406

Jiang, P., Wu, H., Wang, W., Ma, W., Sun, X., and Lu, Z. 2007. MiPred: classification of real and pseudo microRNA precursors using random forest prediction model with combined features. Nucleic Acids Res. 35: W339-W344.

Jouraku, A., Yamamoto, K., Kuwazaki, S., Urio, M., Suetsugu, Y., Narukawa, J., Miyamoto, K., Kurita, K., Kanamori H., Katayose, Y., Matsumoto, T., and Noda, H. 2013. KONAGAbase: a genomic and transcriptomic database for the diamondback moth, *Plutella xylostella*. BMC Genomics 14: 464.

Kozomara, A., and Griffiths-Jones, S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 39: D152-D157.

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. 2003. Prediction of mammalian microRNA targets. Cell 115: 787-798.

Liang, P., Feng, B., Zhou, X., and Gao, X. 2013. Identification and developmental profiling of microRNAs in diamondback moth, *Plutella xylostella* (L.). PLoS ONE 8(11): e78787.

Liu, S., Gao, S., Zhang, D., Yin, J., Xiang, Z., and Xia, Q. 2010. MicroRNAs show diverse and dynamic expression patterns in multiple tissues of *Bombyx mori*. BMC Genomics11: 85

Pasquinelli A. E. 2012. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat. Rev. Genet. 13: 271-282.

Pillai, R. S., Bhattacharyya, S. N., and Filipowicz, W. 2007. Repression of protein synthesis by miRNAs: how many mechanisms. Trends Cell Biol. 17: 118-126.

Rigoutsos, I. 2009. New tricks for animal microRNAs: targeting of amino acid coding regions at conserved and nonconserved sites. Cancer Res. 69: 3245-3248.

Shin, C., Nam, J. W., Farh, K. K., Chiang, H. R., Shkumatava, A., and Bartel, D. P. 2010. Expanding the microRNA targeting code: functional sites with centered pairing. Mol. Cell 38: 789-802.

Singh, J., and Nagaraju, J. 2008. Insilco prediction and characterization of microRNAs from red flour beetle (*Tribolium castaneum*). Insect Mol. Biol. 17: 427-436.

Skalsky, R., Vanlandingham, D. L., Scholle, F., Higgs, S., and Cullen, B. R. 2010. Identification of microRNAs expressed in two mosquito vectors, *Aedes albopictus* and *Culex quinquefasciatus*. BMC Genomics 11: 119.

Tabashnik, B. E., Huang, F., Ghimire, M. N., Leonard, B. R., Siegfried, B. D., Rangasamy, M., Yang, Y., Wu, Y., Gahan, L. J., Heckel, D. G., Bravo, A., and Soberón, M. 2011. Efficacy of genetically modified Bt toxins against insects with different genetic mechanisms of resistance. Nat. Biotechnol. 29: 1128-1131.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731-2739.

VUORINEN, T., NERG, A. M., IBRAHIM, M. A., REDDY, G. V. P., AND HOLOPAINEN, J. K. 2004. Emission of *Plutella xylostella*-induced compounds from cabbages grown at elevated CO_2 and orientation behavior of the natural enemies. Plant Physiol. 135: 1984-1992.

WINTER, J., AND DIEDERICHS, S. 2011. Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. RNA Biol. 8: 1149-1157.

XIE, W., LEI, Y., FU, W., YANG, Z., ZHU, X., GUO, Z., WU, Q., WANG, S., XU, B., ZHOU, X., AND ZHANG, Y. 2012. Tissue-specific transcriptome profiling of *Plutella xylostella* third instar larval midgut. Intl.. J. Biol. Sci. 8(8): 1142-1155

XU, P., GUE, M., AND HAY, B. A. 2004. MicroRNAs and the regulation of cell death. Trends Genet. 20: 617-624

YOU, M. S., YUE, Z., HE, W. Y., YANG, X. H., YANG, G., XIE, M., ZHAN, D. L., BAXTER, S. W., VASSEUR, L., GURR, G. M., DOUGLAS, C. J., BAI, J. L., WANG, P., CUI, K., HUANG, S .G., LI, X. C., ZHOU, Q., WU, Z. Y., CHEN, Q.L., LIU, C. H., WANG, B., LI, X. J., XU, X. F., LU, C. X., HU, M., DAVEY, J. W., SMITH, S. M., CHEN, M. S., XIA, X. F., TANG, W. Q., KE, F. S., ZHENG, D. D., HU, Y. L., SONG, F. Q., YOU, Y. C., MA, X. L., PENG, L., ZHENG, Y. K., LIANG, Y., CHEN, Y. Q., YU, L. Y., ZHANG, Y. N., LIU, Y. Y., LI, G. Q., FANG, L., LI, J. X., ZHOU, X., LUO, Y. D., GOU, C. Y., WANG, J. Y., WANG, J., YANG, H. M., AND WANG, J. 2013. A heterozygous moth genome provides insights into herbivory and detoxification. Nat. Genet. 45(2): 220-225

YU, X., ZHOU, Q., AND LI, S. C. 2008. The silkworm (Bombyx mori) microRNAs and their expressions in multiple developmental stages. PLoS One 3: e2997

ZHANG, B. H., PAN, X. P., CANNON, C. H., COBB, G. P., AND ANDERSON, T. A. 2006. Computational identification of microRNAs and their targets. Comput Biol Chem 30: 395-407

ZHOU, M., WANG, Q., SUN, J., LI, X., XU, L., YANG, H., SHI, H., NING, S., CHEN, L., LI, Y., HE, T., AND ZHENG, Y. 2009. Insilco detection and characteristics of novel microRNA genes in the Equus caballus genome using an integrated ab initio and comparative genomic approach. Genomics 94(2):125-131

ZUKER, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31: 3406-3415