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Research Paper

Identification of MicroRNAs in *Helicoverpa armigera* and *Spodoptera litura* Based on Deep Sequencing and Homology Analysis

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Abstract

The current identification of microRNAs (miRNAs) in insects is largely dependent on genome sequences. However, the lack of available genome sequences inhibits the identification of miRNAs in various insect species. In this study, we used a miRNA database of the silkworm *Bombyx mori* as a reference to identify miRNAs in *Helicoverpa armigera* and *Spodoptera litura* using deep sequencing and homology analysis. Because all three species belong to the Lepidoptera, the experiment produced reliable results. Our study identified 97 and 91 conserved miRNAs in *H. armigera* and *S. litura*, respectively. Using the genome of *B. mori* and BAC sequences of *H. armigera* as references, 1 novel miRNA and 8 novel miRNA candidates were identified in *H. armigera*, and 4 novel miRNA candidates were identified in *S. litura*. An evolutionary analysis revealed that most of the identified miRNAs were insect-specific, and more than 20 miRNAs were Lepidoptera-specific. The investigation of the expression patterns of *miR-2a*, *miR-34*, *miR-2796-3p* and *miR-11* revealed their potential roles in insect development. miRNA target prediction revealed that conserved miRNA target sites exist in various genes in the 3 species. Conserved miRNA target sites for the *Hsp90* gene among the 3 species were validated in the mammalian 293T cell line using a dual-luciferase reporter assay. Our study provides a new approach with which to identify miRNAs in insects lacking genome information and contributes to the functional analysis of insect miRNAs.

Key words: microRNA; deep sequencing; homolog analysis; *Helicoverpa armigera*; *Spodoptera litura*; *Bombyx mori*.

Introduction

MicroRNAs (miRNAs) are approximately 22 nucleotide (nt) long, single-stranded, endogenous, small non-coding RNAs. miRNAs are processed into double-stranded complexes by Drosha and Dicer from hairpin precursors transcribed from the genome by RNA polymerase II. One of the strands binds to Argonaute to form an RNA-induced silencing complex (RISC), which guides the complex to target mRNAs to direct translational silencing or mRNA degradation [1, 2]. The remaining strand, called the miRNA-star strand (miR*), either is degraded or accumulates at low levels in most cases [3].

In recent years, numerous miRNAs have been identified in plants, animals, and viruses [4-6]. In miRBase Release 18 (http://www.mirbase.org/, released on November, 2011), the miRNAs of 168 spe-

cies were published, including 24 insect species. Although miRNAs such as *cel-let-7* and *cel-lin-4* were identified by a forward genetic method [7-9], most miRNAs have been identified by a combination of RNA sequencing and *in silico* prediction methods [10, 11]. However, the lack of sufficient genome information in most species, especially in non-model species, has limited the further identification of a wider range of miRNAs.

The cotton bollworm (*Helicoverpa armigera*) and the cotton leafworm (*Spodoptera litura*) are serious lepidopteran pests of various crops. Because miRNAs are involved in regulating development and metamorphosis in insects [4, 12-18], the identification of miRNAs in these two pests will help to uncover their physiological roles and provide potential targets for pest control. In *S. litura*, many miRNAs have been identified by computational prediction or stem-loop polymerase chain reaction (PCR) [19, 20]. However, deficient genome sequences have restricted the further identification of miRNAs. Additionally, no miRNAs have been identified through small RNA sequencing in *H. armigera* and *S. litura*. In contrast, the silkworm *Bombyx mori*, a model lepidopteran insect, has a previously released genome sequence with more than 500 mature miRNA sequences published. Although miRNAs are frequently acquired and lost during evolution, functional miRNAs evolve slowly, maintaining a particularly high homology among closely related species [21-23]. The relatively close relationship among *H. armigera*, *S. litura* and *B. mori* increases the likelihood of identifying conserved miRNAs in *H. armigera* and *S. litura* based on *B. mori* miRNA and genomic sequences.

In this study, we sequenced small RNAs (sRNAs) of *H. armigera* and *S. litura*, and identified 97 and 91 conserved miRNAs, respectively, using *B. mori* miRNAs as references (named har-miRNAs for miRNAs from *H. armigera* and sli-miRNAs for miRNAs from *S. litura*). We also identified 1 novel har-miRNA, 8 har-miRNA candidates and 4 sli-miRNA candidates by computational prediction based on the *B. mori* genome and *H. armigera* BAC sequences. A comparison of these miRNAs with those from other species indicated that most of the identified miRNAs were insect-specific, with many being Lepidoptera-specific. Quantitative reverse transcription PCR (qRT-PCR) was performed to investigate the expression profiles of 4 miRNAs, and the results revealed their potential roles in insect development. miRNA target prediction revealed that conserved miRNA target sites exist in various genes among the 3 species. In addition, 3 conserved miRNA targets of the *Hsp90* gene were validated in a mammalian 293T cell line using a dual-luciferase reporter assay. The present study not only provides a new strategy for miRNA identification in insect species lacking genome information, but also presents insights into the conservation and functions of lepidopteran miRNAs.

Materials and Methods

Insect strains

H. armigera and *S. litura* strains were maintained in our laboratory at the Shanghai Institute of Plant Physiology and Ecology and were reared on an artificial diet under a 28°C temperature and a Light-Dark 14:10 photoperiod.

Small RNA sequencing

Total RNA was isolated from whole-body homogenates of different developmental stages of *H. armigera* and *S. litura* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Small RNAs were sequenced with an Illumina Genome Analyzer (Illumina, SanDiego, CA, USA) at the Beijing Genomics Institute (BGI, Shenzhen, Guangdong, China). First, total RNA was size-fractionated and 10–30 nt sRNAs were isolated. Subsequently, 5' and 3' adaptors were ligated to the sRNAs, and double-stranded nucleic acids were acquired after reverse transcription PCR (RT-PCR). The PCR products were sequenced using Solexa's sequencing-by-synthesis (SBS) technology, which is a high-throughput sequencing method. The 35 nt sequence tags from the Solexa sequencing were subjected to a primary analysis in which low-quality tags and adaptor contaminants were discarded.

miRNA prediction

Using the *B. mori* miRNAs presented in miRBase Release 18 (http://www.mirbase.org/) as references, sequences that aligned perfectly with precursor miRNAs and aligned to mature miRNAs with forward matching were annotated as conserved miRNAs in *H. armigera* and *S. litura*. To identify other types of sRNAs, we used sequences from SilkDB (SilkDB, http://silkworm.genomics.org.cn/) [24, 25], NCBI (http://www.ncbi.nlm.nih.gov/), Rfam (http://rfam.sanger.ac.uk/) and RepBase (http://www.girinst.org/repbase/) as references to annotate the non-coding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs and repeat-associated small RNAs) and degraded fragments of expressed genes (exons and introns) in the remaining sequences. The BLASTn program was used as the alignment method, and the e-value was set to 10^{-5}. Using the miRNA

prediction software Mireap (http://sourceforge.net/projects/mireap/), we predicted novel miRNAs from the remaining sRNAs. *B. mori* genomic sequences and *H. armigera* BAC sequences were used as references to provide flanking sequences of the sRNAs for fold-back structure prediction. sRNAs that could not be annotated were classified as unannotated.

Homology analysis of miRNAs

The miRNA sequences in the other species were downloaded from miRBase (Release 18) and the relevant published studies [26-28]. BLASTn was used to compare the *H. armigera* and *S. litura* miRNAs with other species. Because all species published in miRBase were analyzed, including species with long evolutionary distances, the e-value was set to 10^{-3} so that more miRNA homologs could be identified. Reverse-matched sequences were discarded from the BLAST results. The alignments of miRNA sequences were conducted by Mega 4 [29], using IUB as the DNA weigh matrix.

Expression profiles of miRNAs

The expression profiles of *miR-2a*, *miR-34*, *miR-2796-3p* and *miR-11* were investigated by qRT-PCR with the miScript Reverse Transcription Kit and a miScript SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. The monitoring and analysis of the qRT-PCR were performed on an ABI 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA), and the PCR conditions were as follows: 95°C for 15 minutes for denaturation and 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds. The primers for each miRNA are listed in Supplementary Material: Table S1. The small nuclear RNA *U6* of *B. mori* was used as an internal control.

Homologous miRNA target prediction

The mRNA sequences for *H. armigera* and *S. litura* genes were downloaded from NCBI. The 3' untranslated regions (UTRs) were extracted for homology analysis. BLASTn was used to find homologous regions in these 3' UTRs, and the e-value threshold was set to 10^{-5}. The miRNA targets were then predicted in the homologous sequences. 3 miRNA target-prediction software programs were used: miRanda (http://www.microrna.org/microrna/getDownloads.do) [30, 31], PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_exe.html), and microTar (http://tiger.dbs.nus.edu.sg/microtar/) [32]. The thresholds were set to a score of ≥ 140 for miRanda (default), ddG ≤ 0 for PITA, and energy ≥ 0.5 for microTar. The miRNA targets predicted by at least two of the software programs were selected. Homologous regions in different species targeted by common miRNAs were considered to contain conserved miRNA target sites.

Plasmid construction and dual-luciferase reporters assay

Precursors of *bmo-miR-14*, *bmo-miR-2766* and *bmo-miR-9a* were amplified (primers listed in Supplementary Material: Table S1) from the *B. mori* genome DNA with 150-300 bp (base pair) flanking sequences and inserted into the multi-cloning site of pmR-mCherry (Clontech, Mountain View, CA, USA) for miRNA expression. We only cloned *B. mori* miRNAs because they have high homology with the corresponding miRNAs of *H. armigera* and *S. litura*. To estimate the regulation of miRNAs at their target sites, the plasmid phRL-TK (Promega, Madison, WI, USA) was used as a reporter. We amplified the 3' UTRs of *BmoHsp90*, *HarHsp90* and *SliHsp90* (primers listed in Supplementary Material: Table S1) and inserted the sequences behind the Renilla luciferase reporter gene in phRL-TK. pGL3 (Promega, Madison, WI, USA) with the Firefly luciferase reporter gene was used as a control to indicate the transfection efficiency.

The mammalian 293T cell line used for the dual-luciferase reporter assay was kindly provided by Dr. Mofang Liu of the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences. The cells were cultured in DMEM (high glucose, Gibco, Life Technologies, Grand Island, NY, USA) with 10% FBS. Before transfection, the cells were plated in a 96-well tissue-culture plate. After 24 h (hours), two reporter plasmids (in total 10 ng per well) and the miRNA-expressing plasmids (100 ng per well) were co-transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Life Technologies, Grand Island, NY, USA). Approximately 24-48 h after the transfection, the Renilla and Firefly luciferase activities were quantified using the Luciferase Assay Reagent II (LAR II) (Promega, Madison, WI, USA) on a Modulus™ single-tube multimode reader (Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturers' instructions. The transfections were performed in triplicate. A paired t-test was used to analyze the significance of the differences.

Results

Identification of conserved miRNAs

Using high-throughput sequencing, we obtained 5,266,540 total sRNA sequences in *H. armigera*, and after eliminating the redundant sequences, we obtained 1,125,767 unique sequences. In *S. litura*,

11,443,493 total sRNA sequences were acquired, corresponding to 901,369 unique sequences. The length of these sRNAs ranged from 10 nt to 30 nt. Their length distributions in both *H. armigera* and *S. litura* showed a bimodal distribution. One of the peaks was at approximately 20–22 nt, representing the miRNAs [33], and the other was at approximately 27 nt, which may be the piRNA-like sRNAs [34] (Fig. 1A, 1B). A comparison between the two species showed that only 2.02% of the unique sRNAs are common between these two organisms; however, these shared sRNAs represent 46.34% of the total sRNAs (Fig. 1C and 1D), which means their average number of reads are higher than the sRNAs specific in each species. Such a redundancy of common sRNAs indicates that those sRNAs occurring simultaneously in both insects were expressed much more abundantly than the species-specific sRNAs.

The acquired sRNAs were aligned with known miRNAs, rRNAs, tRNAs, snRNAs, snoRNAs, repeat associated RNAs, introns, and exons. The sRNAs aligned with *B. mori* miRNA precursors and mature sequences in miRBase Release 18 [35-38] were annotated as miRNAs. The distributions of the sRNAs among different categories are shown in Supplementary Material: Table S2. After alignment, we obtained 923 and 818 unique sRNAs in *H. armigera* and *S. litura*, respectively, aligned to 75 known miRs and 25 known miR*s in *B. mori* (Table 1). Among the sequences aligned to each *B. mori* miRNA, only the one with the most reads was retained as the final version. We finally acquired 97 conserved miRNAs in *H. armigera* and 91 in *S. litura* (Supplementary Material: Table S3).

Table 1. Known miRNA alignment.

	miRs	miR*s	miRNA precursors
In Total	75	25	78
H. armigera	74	23	77
S. litura	71	20	74

All the *B. mori* miRNAs in miRBase Release 18 were used as the references. The row "In Total" shows the information about the alignment of all the miRNAs in both *H. armigera* and *S. litura*. The three columns summarized the numbers of miRNA sequences from each species aligned to *B.mori* miRs, miR*s, or miRNA precursors.

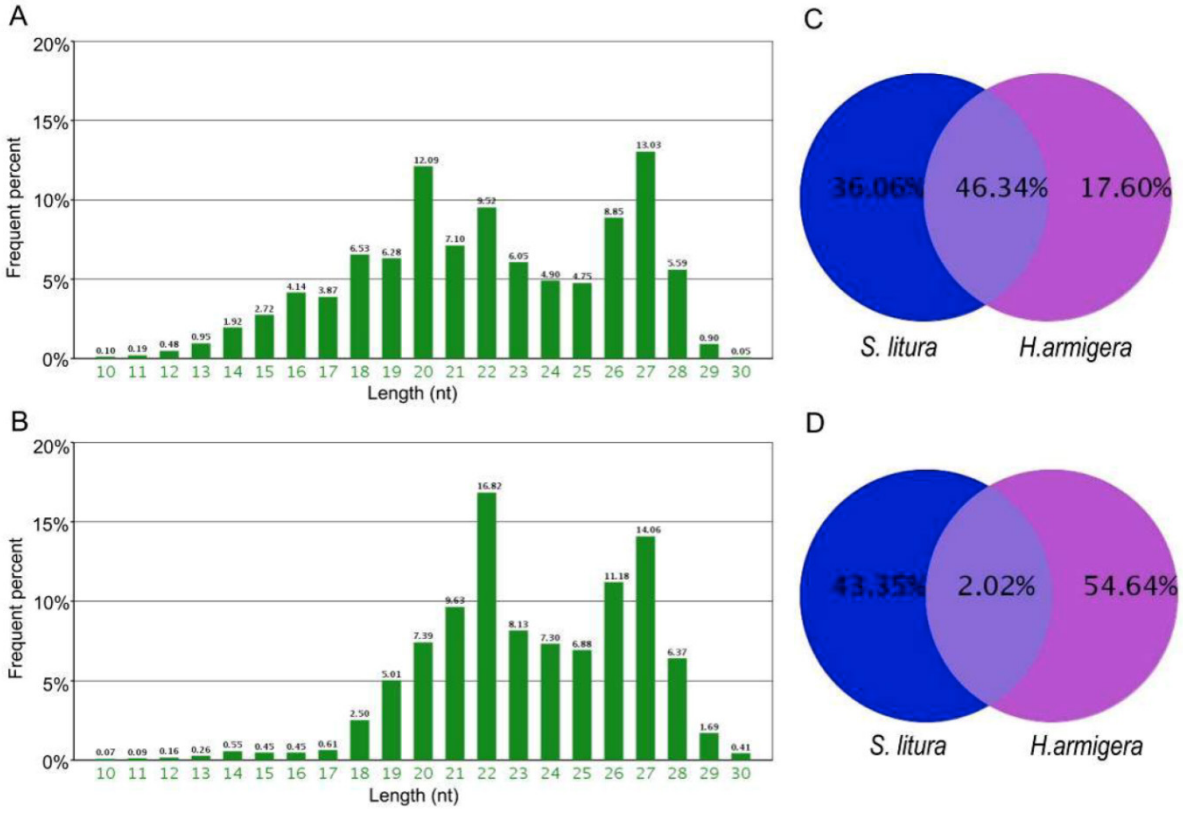

Figure 1. Length distribution and comparison of small RNA tags sequenced in *H. armigera* and *S. litura*. The two bar diagrams show the length distribution of the small RNAs in *H.armigera* (A) and *S. litura* (B). Comparison of the total (C) and unique (D) small RNAs are presented.

Novel miRNA prediction

Here we used BAC sequences of *H. armigera* and the genomic sequence of *B. mori* to perform the novel miRNA prediction because no genomic information for *H. armigera* or *S. litura* was available and no *S. litura* BAC sequences were found in NCBI. Using mireap, we identified 10 possible mature miRNAs in *H. armigera* and 4 in *S. litura* (Table 2). Among them, *har-miR-m1*, *har-miR-m2* and the 4 *S. litura* candidate miRNAs were identified from the *B. mori* genomic sequences, and the other 8 sequences of *H. armigera* were identified from the *H. armigera* BAC sequences. Only *har-miR-m7* had more than 5 reads, and its complementary miR* was also identified, indicating that it was a novel miRNA. All the other sRNA sequences did not have corresponding miR*s that were identified and thus were classified as miRNA candidates. However, *har-miR-m1* and *sli-miR-m1* were homologous to each other and thus they may be a common miRNA in these 2 species, with their miR* sequences most likely being expressed at a level too low for detection. In addition, *har-miR-m2* can be aligned to *miR-193* in some insects other than *B. mori*, including *Heliconius melpomene*, which is a lepidopteran species, indicating that this miRNA may also be conserved in insects.

Nucleotide bias analysis of identified miRNAs

The nucleotide bias at the first position of the identified miRNAs with a certain length was analyzed (Fig. 2 A, B). In both *H. armigera* and *S. litura*, the miRNAs showed a dominant bias to uracil (U) at the first nucleotide, especially the miRNAs with a length of 19-23 nt. This observation agrees with the characteristic that miRNAs usually begin with a U at the 5' terminus [39].

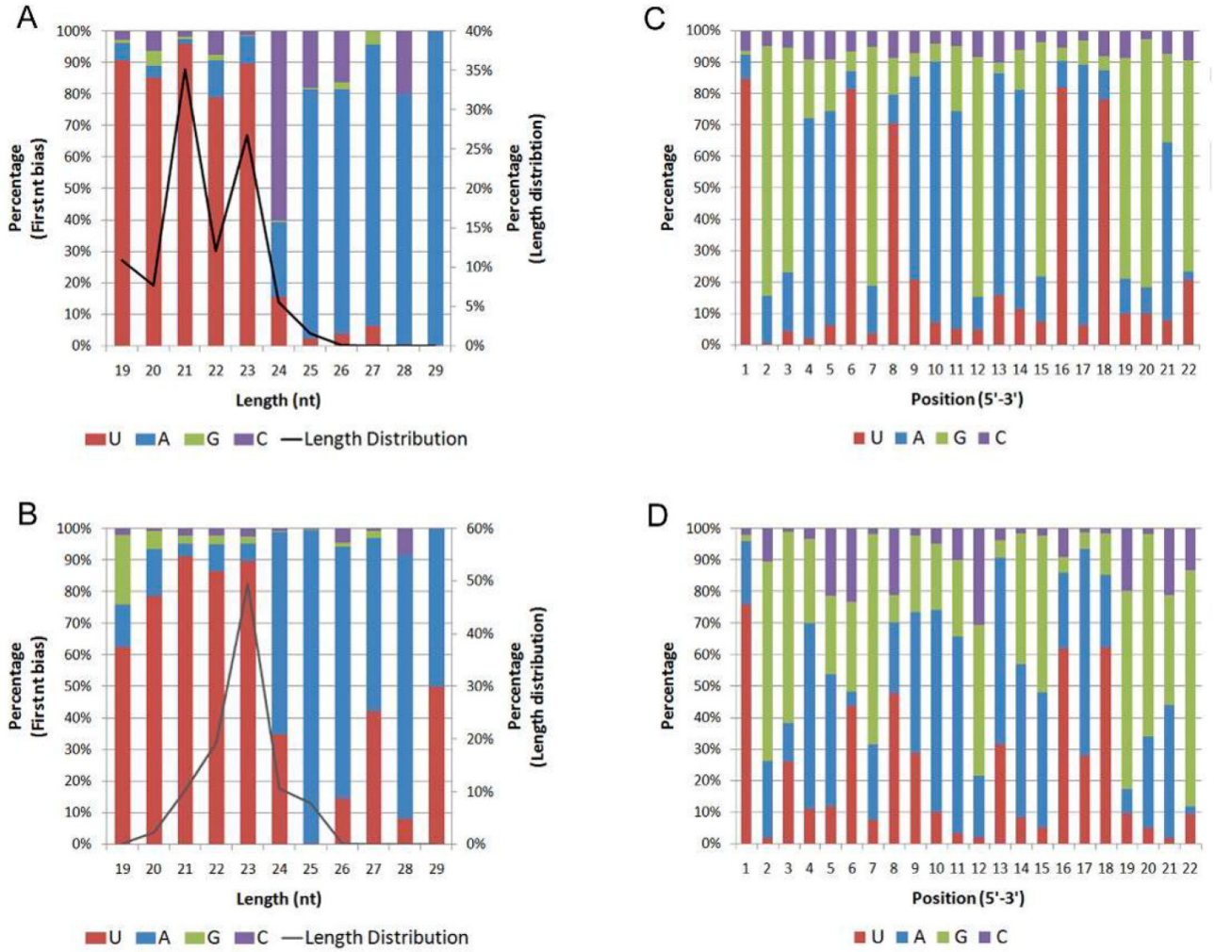

Figure 2. First nucleotide bias and position nucleotide bias. The first nucleotide bias of the miRNAs in *H. armigera* (A) and *S. litura* (B) was analyzed in combination with the length distribution. In addition, the miRNA nucleotide bias at each position in *H.armigera* (C) and *S. litura* (D) was examined.

Table 2. Novel miRNA sequences and numbers of reads in *H. armigera* and *S. litura*.

miRNA	Sequences	Length (nt)	Number of reads
har-miR-m1	GUGGGGCAAAUUGCGAAAGC	20	16
har-miR-m2	UACUGGCCUGCUAAGUCCCAA	21	43
har-miR-m3	GAUCAACUUUCGACUGAUUUUUC	23	6
har-miR-m4	UCCACAGUACCUGGCGUGUCCGCU	24	9
har-miR-m5	UGACAUCUCUUUGAGGAGCGCGUG	24	5
har-miR-m6	AGCAGUGGGACGAUAAAAAGG	21	5
har-miR-m7	UCUCACGGAUAUAAAAUUUAAGA	23	7
*har-miR-m7**	UGUGUCCGUUUUUACAACAAGC	22	1
har-miR-m8	ACAAAGCGAUUUCGGCCCGAC	21	4
har-miR-m9	UGUCCGCACAAUGGUGACACCCG	23	3
sli-miR-m1	GUGGGGCAAAUUGCGAAAGCUG	22	14
sli-miR-m3	CUCUCACCCAGGAGGCUCGG	20	20
sli-miR-m4	UACAGCUAGGAUACCAAAGAGA	22	14
sli-miR-m5	CGGGAGAUGUGGUGUUCGGGA	21	336

We also analyzed the percentage of the four nucleotides appearing at each position (Fig. 2 C, D). The five positions showing the most dominant bias to U are the 1st, 6th, 8th, 16th, and 18th nucleotides. In canonical cases, the 2nd to the 8th nucleotides of miRNAs, called the "seed region," pair perfectly with their target sites. Some nucleotides in the 3' portion of many miRNAs also show supplementary pairing [40]. Thus, the 1st and 8th nucleotides are at the edges of the "seed region," the 6th nucleotide is within the "seed region," and the 16th and 18th nucleotides are near the 5' terminus of the miRNAs. The bias to U at these positions may contribute to the miRNA regulatory mechanism. The nucleotide bias of these miRNAs was quite similar to that described in a previous report on miRNAs [33].

Homology analysis of identified miRNAs

In miRBase Release 18, 562 *B. mori* miRNAs are included. Among these, only 100 miRNAs were identified in *H. armigera* and/or *S. litura*, with 88 being common to all three species (Fig. 3A). The homology of these miRNAs in insects and other animal classes was analyzed. We used BLASTn to align the *H. armigera* miRNAs (har-miRNAs) and *S. litura* miRNAs (sli-miRNAs) to all miRNA sequences of other species in miRBase (Release 18), and to the published miRNA sequences in other lepidopteran insects [26-28]. Only metazoan species contain homologs of these miRNAs. We divided the species into four categories: insects, other arthropods (arthropods other than insects), other invertebrates (invertebrates other than arthropods) and vertebrates. Based on the BLAST results, the miRNAs were classified into four types: highly conserved (with homologs in vertebrates), invertebrate-specific (with homologs only in invertebrates), arthropod-specific (with homologs only in arthropods) or insect-specific (with homologs only in insects). 16 miRNAs were highly conserved: *let-7*, *miR-10*, *miR-100*, *miR-124*, *miR-133*, *miR-137*, *miR-184*, *miR-190*, *miR-1a*, *miR-210*, *miR-281*, *miR-33*, *miR-7*, *miR-993a**, *miR-993b**, and *miR-9a*. However, most of the miRNAs were insect-specific. Among those, 29 har-miRNAs and 20 sli-miRNAs had homologs only in lepidopteran species, and thus were considered possible Lepidoptera-specific miRNAs (Supplementary Material: Tables S4 and S5).

Although occasionally expressed abundantly, miR*s are often degraded or show a low expression level [3, 41, 42]. Therefore, we assumed that miR*s vary more dramatically than miRs among different species. Consistent with our hypothesis, most miR*s were insect-specific (Supplementary Material: Tables S4 and S5). Surprisingly, there were two miR*s in the highly conserved miRNAs, namely, *miR-993a** and *miR-993b**. However, in the BLAST results of these two miR*s, only *miR-10* family members in vertebrates were homologous (Fig. 3B). In fact, there was no *miR-993* in vertebrates. In *H. armigera* and *S. litura*, *miR-10*s were also homologous to *miR-993a**s and *miR-993b**s. Therefore, the *miR-993*s in invertebrates may be mimics of *miR-10*s and are most likely in the same miRNA family as *miR-10*s.

Expression analysis of identified miRNAs

qRT-PCR was used to validate and analyze the expression of two conserved miRNAs – *miR-2a* and *miR-34* – and two insect-specific miRNAs – *miR-2796-3p* and *miR-11*. Because the latter 2 miRNAs are expressed remarkably differently between the 2

species, we only chose the species with the higher expression level to analyze the expression profile. Although the expression levels were quite different, all 4 miRNAs could be detected in the relevant species, validating the sequencing results (Fig. 4).

We selected 4 developmental stages – early larva, late larva, pupa and adult – in which to analyze the expression profile. The miRNAs could be detected during every stage of the corresponding species, except that *har-miR-2796-3p* was not detected in the *H. armigera* pupal stage, which was most likely due to its low expression level at this stage. All of these miRNAs showed stage-specific expression profiles during development. *miR-2a* showed a higher expression at the pupal stage in both species, indicating its possible functions at this stage. The expression of *miR-34* was more abundant during the early larval and adult stages in both species; however, its level was highest in adults in *H. armigera*, whereas its expression reached its peak in early larvae in *S. litura*. *har-miR-2796-3p* was expressed most abundantly in adults, and *sli-miR-11* was highly expressed in the early larval and pupal stages.

Conserved miRNA targets

Using 3 miRNA target prediction software programs – miRanda, PITA, and microTar – we predicted the targets of conserved miRNAs in homologous regions in the 3' UTRs of corresponding genes in *B. mori*, *H. armigera* and *S. litura*. The common miRNA targets are listed in Table 3. 19 different genes are involved, including transcription factors, heat shock proteins, and genes involved in hormone pathways and metabolism. The targets predicted by all 3 programs are considered most reliable. Moreover, the miRNA targets can be regarded as highly conserved if they exist in the homologous gene regions of all 3 species. The results show that 3 miRNA targets are highly conserved. All the 3 targets exist in the 3' UTR of *Hsp90*, and the targeting miRNAs are *miR-9a*, *miR-14* and *miR-2766* (Fig. 5A). Using dual-luciferase reporter plasmids to transfect 293T cells, we validated these target sites in the *Hsp90* 3' UTR. When we over-expressed *bmo-miR-9a*, *bmo-miR-14* and *bmo-miR-2766*, the relative expression of the reporter gene (Renilla luciferase) exhibited a significant decrease, with a suppression efficiency from approximately 20% to 45% (Fig. 5B). Suppression of *bmo-miR-2766* on *SliHsp90* is slightly less significant (*p-value*=0.05104), probably due to the subtle differences between *sli-miR-2766* and *bmo-miR-2766*. Because most miRNAs in animals function in the fine tuning of their target genes [43], a gene down-regulated by miRNAs even at an efficiency of less than 20% can be regarded as a target [44]. Therefore, our results demonstrate the regulation of *Hsp90*expression by these miRNAs.

Table 3. Common miRNAs targeting the homologous regions in 3' UTRs of *B. mori*, *H. armigera*, and *S. litura*.

Gene	ID_har*	Homologous Region In 3'UTR (har)§	ID_sli*	Homologous Region In 3'UTR (sli)§	ID_bmo*	Homologous Region In 3'UTR (bmo)§	Common miRNA Targets¶
14-3-3zeta	GQ131301	6..249			AB378097	6..243	*miR-263a*
		6..296			DQ311235	6..291	*miR-278**
					EF210316	6..291	
					NM_001046699	6..291	
actin	EU527017	58..141			AB701689	50..132	*bantam, miR-965**
					NM_001126254	50..132	
ADP/ATP translocase	AY253868	1..28			AY227000	1..28	*miR-2765*
					NM_001043607	1..28	
ArgK	EF600057	1..211	HQ840714	1..211			*miR-8*
beta-tubulin	JF767013	100..152			AB003287	105..157	*miR-2796-5p*
					AB072307	106..158	
					NM_001043422	106..158	
CCE	FJ997310	496..541	AB521203	162..207			*miR-87*
chitinase	AY325496	780..976	AB032107	860..1055			*miR-87*
			AY325497	861..1056			
E75			JQ266225	22..319	AB024904	21..314	*miR-33, miR-278*, miR-87*
					AB024905	21..314	
					NM_001112609	21..314	
					NM_001112610	21..314	

				3..319	AF332550	3..316	*miR-33, miR-278**
					AF332551	3..316	
					NM_001043577	3..316	
EcR			EU180021	14..51	BMOBMECRB1	14..51	*miR-14, miR-9a**
					D49476	14..51	
					D49478	14..51	
			HM046618	117..154	NM_001043866	14..51	
					NM_001173375	14..51	
					BMOECDYREC	695..732	
FF1			HQ260326	77..175	AB649122	82..180	*miR-305**
					AF426830	82..180	
					NM_001044063	82..180	
			HQ260326	77..275	BMOFTZF1P	15..203	*miR-305*, miR-970*
Hsp21.4			HM046612	106..148	AB195972	109..154	*miR-1175-3p*
					NM_001043520	109..154	
			HM046612	581..662	AB195972	568..647	*miR-305*
					NM_001043520	568..647	
Hsp70	FJ432703	25..85	HM046611	27..87			*miR-8*
	JF417984	497..624			AB016836	370..500	*miR-1a*, miR-3338*
					FJ573459	370..500	
					NM_001043372	370..500	
Hsp90	FJ986209	9..101	HM046609	12..101			*miR-14, miR-2766, miR-285, miR-998, miR-9a, miR-9c**
	HM593517	9..101					
	FJ986209	57..108			AB060275	43..96	*miR-14, miR-2766, miR-9a, miR-9c**
	HM593517	57..108			NM_001043411	43..96	
			HM046609	57..98	AB060275	43..84	*miR-14, miR-2766, miR-9a, miR-9c**
					NM_001043411	43..84	
LpR			GU433377	30..69	AB211594	30..69	*miR-13a*
			GU433377	1304..1375	AB211594	1048..1116	*miR-iab-8*
			GU433377	1343..1375	AB201471	1079..1111	*miR-iab-8*
					AB201472	1079..1111	
					AB201473	1079..1111	
					AB201474	1654..1686	
					NM_001111338	1079..1111	
					NM_001111339	1079..1111	
					NM_001111340	1079..1111	
PBANR	JN228350	479..596			JN228346	843..960	*miR-375**
TCTP	GU969276	33..152	HQ896486	31..149			*miR-2766, miR-305*, miR-989*
	GU969276	121..154			DQ003481	99..132	*miR-989, miR-14, miR-2766, miR-375*, miR-9c*
					EF210320	99..132	
					NM_001044107	99..132	
			HQ896486	118..149	DQ003481	99..130	*miR-989, miR-14, miR-2766, miR-9c*
					EF210320	99..130	
					NM_001044107	99..130	
TF AP-4	DQ224406	20..91			EU534187	21..92	*miR-9a**
					NM_001123344	21..92	
TF POU	AY513764	549..675			BMOPOUM1	494..619	*miR-2745, miR-316**
USP	EU526832	467..552	EU180022	125..212			*miR-79, miR-9a**

***:** "ID" indicates the "LOCUS" field in NCBI. Abbriviation: har: *H. armigera*; sli: *S. litura*; bmo: *B. mori*.

§: BLASTn was used to identify homologous regions in 3' UTRs. E-value threshold was set to 10^{-5}.

¶: miRanda, PITA, and microTar were all used for miRNA target prediction. Only the miRNA targets being predicted by more than two software programs are included in this table.

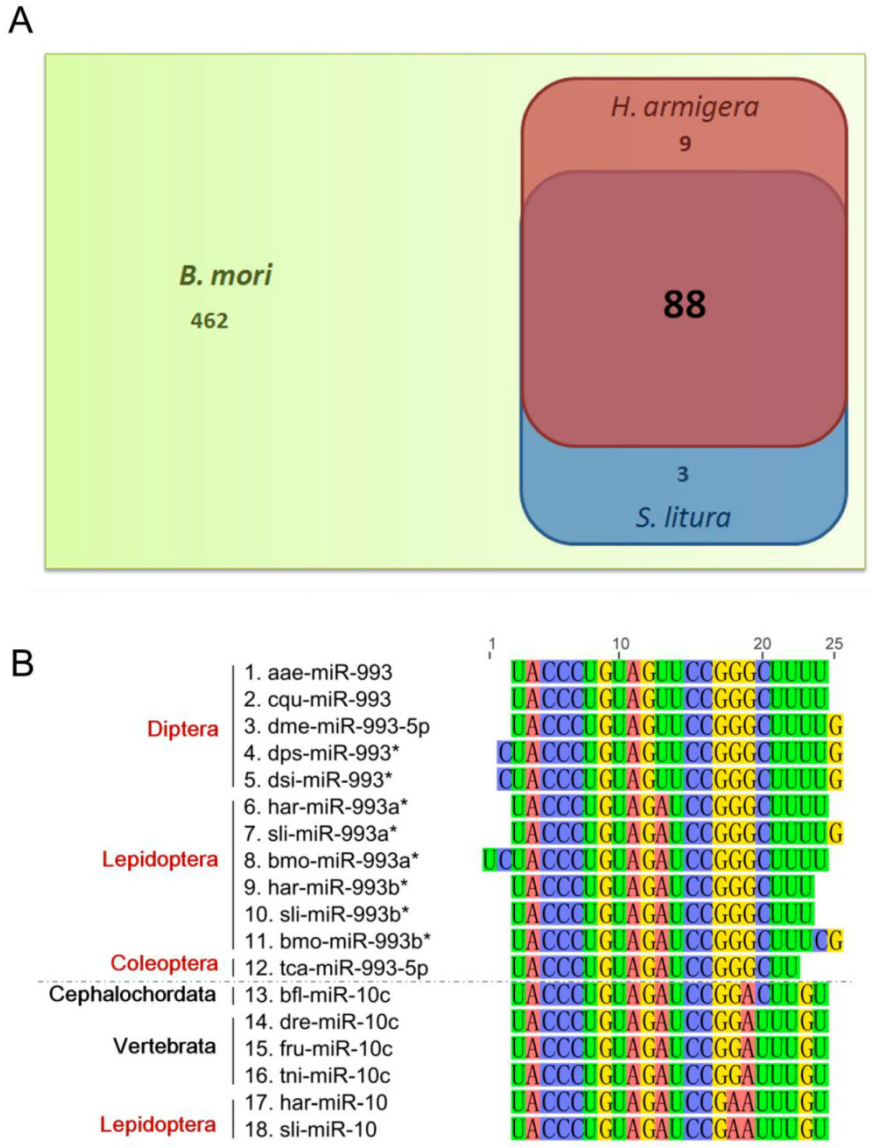

Figure 3. Homology analysis of the identified miRNAs. (A) Comparison of identified miRNAs of *H. armigera* (red) and *S. litura* (blue) as well as those of *B. mori* (green). (B) Homology analysis of miRNA sequences homologous to lepidopteran *miR-993a** and *miR-993b**. The alignment was conducted for all the homologs of *har-miR-993a**, *har-miR-993b**, *sli-miR-993a**, and *sli-miR-993b**. The nucleotides are marked in different colors. The names of the miRNA sequences are listed on the left, labeled with their taxonomic order (for insects, marked in red) or phylum (for non-insects, marked in black). The abbreviations represent the species as follows: aae, *Aedes aegypti*; cqu, *Culex quinquefasciatus*; dme, *Drosophila melanogaster*; dps, *Drosophila pseudoobscura*; dsi, *Drosophila simulans*; har, *Helicoverpa armigera*; sli, *Spodoptera litura*; bmo, *Bombyx mori*; tca, *Tribolium castaneum*; dre, *Danio rerio*; fru, *Fugu rubripes*; tni, *Tetraodon nigroviridis*. The dotted line in the middle of the figure separates the *miR-993* family members from the *miR-10* family members.

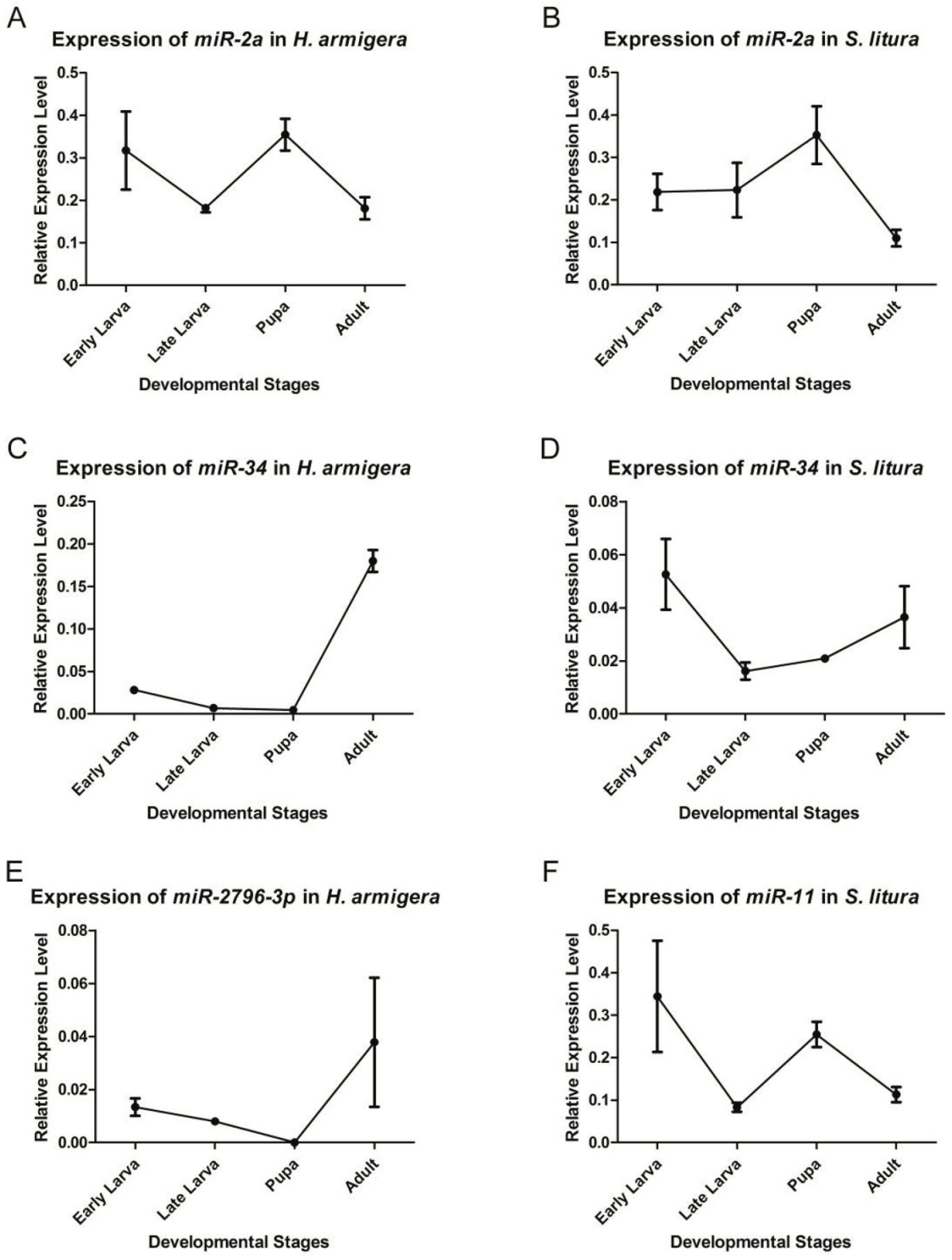

Figure 4. Expression levels of miRNAs at different developmental stages. qRT-PCR was used to analyze the expression levels of *miR-2a* (A, B), *miR-34* (C, D), *har-miR-2796-3p* (E) and *sli-miR-11* (F). The transcript levels were calculated relative to the amount of *U6* after normalization. 3 technical replications were performed. The values are the average±SD of the 3 repeats.

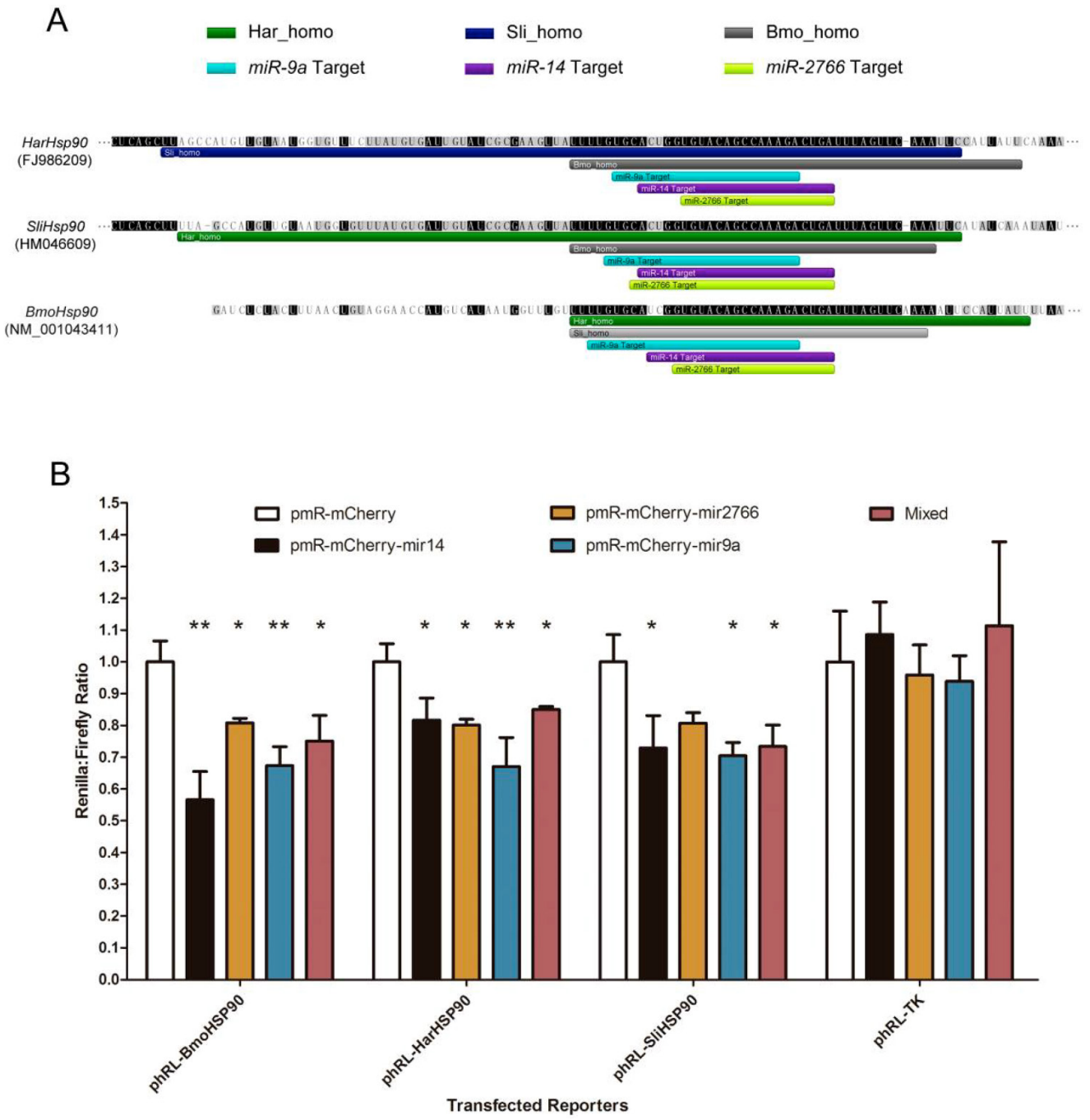

Figure 5. Conserved miRNA target sites in 3' UTRs of *Hsp90* genes. (A) Homologous regions and miRNA target sites in the 3' UTRs of *Hsp90* genes. The bars indicate regions homologous to *H. armigera* (dark green), *S. litura* (dark blue) or *B. mori* (grey) and predicted miRNA target sites of *miR-9a* (light blue), *miR-14* (purple) or *miR-2766* (light green). (B) Down-regulation of reporter gene expression with the 3' UTRs of *Hsp90* genes. miRNA precursors were inserted into pmR-mCherry for miRNA over-expression. These miRNA-expressing plasmids were co-transfected into 293T cells with the pGL3-promoter and pRL-TK parental (Renilla control) or pRL-TK-3'UTR plasmids into which the *Hsp90* 3' UTRs were respectively cloned. The *y*-axis shows the Renilla luciferase activity (relative luminescence units [RLU]) normalized to the firefly luciferase activity and compared with the vector control, which was set to 1.0 within each experiment. The values are the average±SD of 3 biological repeats. The abbreviations represent the species as follows: Bmo, *B. mori*; Har, *H. armigera*; Sli, *S.litura*. A paired *t-test* was used to analyze the significance of the differences. A difference with *p-value* less than 0.05 was considered significant (marked with "*" in the figure) and less than 0.01 was considered highly significant (marked with "**").

Discussion

Homology-based predictions of lepidopteran miRNA using the *B. mori* genome sequence is a valid approach

The identification of miRNAs based on genomic informations has been reported in several lepidopteran insects, such as silkworm (*B. mori*) [45, 46], tobacco hornworm (*Manduca sexta*) [28] and monarch butterfly (*Danaus plexippus*) [27]. Additionally, in two lepidopteran insects – *Heliconius melpomene* and *Bicyclus anynana* – miRNAs have been identified using BAC sequences [26, 47]. In the present study, we report the identification of miRNAs in *H. armigera* and *S. litura* based on small RNA sequencing. Because complete *H. armigera* or *S. litura* genomes are not available, we used the *B. mori* miRNA and genome sequences as references. As a result, 33.66% and 33.34% of the sRNA sequences in *H. armigera* and *S. litura*, respectively, could be mapped to the *B. mori* genome. Although the percentage is lower than that of mapped *B. mori* sRNA sequences (which is greater than 60%) [45, 46], the homology is still significant. The lower mapping ratio may be because *B. mori* belongs to Bombycidae, whereas *H. armigera* and *S. litura* belong to Noctuidae. The miRNAs identified in this study are believed to be highly conserved among lepidopteran insects.

Most of the conclusions of the present study are based on the assumption that the mature miRNA sequences are conserved among related species. To evaluate the reliability of this assumption, we chose two highly conserved har-miRNAs – *har-let-7* and *har-miR-133* – and two insect-specific har-miRNAs – *har-miR-1000* and *har-miR-14* – to analyze the conservation among the homolog precursors (Supplementary Material: Fig. S1). Insects from different orders published in miRBase were considered in this analysis. The results demonstrated that the precursors were conserved in the mature sequence region and that two nucleotides flanking this region were also relatively conserved, particularly among species belonging to the same order. The precursors of the miRNA homologs from species in the same genus were more analogous. These results are in accordance with a previously published study on highly conserved miRNAs in *S. litura* [19]. Thus, when *B. mori* pre-miRNAs and mature miRNAs are used as references for lepidopteran miRNA identification, conserved miRNAs in *H. armigera* and *S. litura* can be obtained.

A minor portion of the identified miRNAs are highly conserved from insects to vertebrates

To understand the homology of miRNAs, we compared the conserved miRNAs of *H. armigera* and *S. litura* with the miRNA sequences of all species published in miRBase (Release 18) and other published lepidopteran miRNAs [26-28]. 16 miRNAs – including *let-7*, *miR-10*, *miR-100*, *miR-124*, *miR-133*, *miR-137*, *miR-184*, *miR-190*, *miR-1a*, *miR-210*, *miR-281*, *miR-33*, *miR-7*, *miR-993a**, *miR-993b**, and *miR-9a* – were conserved from insects to vertebrates (Supplementary Material: Tables S4 and S5). These miRNAs are thought to have homologous functions in diverse species. Studies on their functions in other species, such as in flies and mice, can provide clues to their functions in lepidopteran insects.

For example, *let-7* has been demonstrated to be involved in the development of diverse animals, including nematode, fly, mouse, and human [48]. In addition, some of the target genes of *let-7* appear to be conserved among these animals, such as the conservation of the interaction of *let-7* and two genes – *RAS* (a homolog of *let-60*) and *TRIM71* (a homolog of *lin-41*) – from nematodes to vertebrates [48]. Moreover, *let-7* is induced by ecdysone [49] and is required for neuromusculature remodeling during the metamorphosis of flies [50]. Therefore, *let-7* in lepidopteran insects may also play a role in developmental regulation. These data provide a hint for the functional analysis of *let-7*.

It was surprising to find two miR*s, *miR-993a** and *miR-993b**, in this highly conserved set of miRNAs. These miR*s are homologous to *miR-10*s in vertebrates. With regard to miRBase, we found that *mir-993*s have only been identified in invertebrates, most of which also expresses *miR-10*s (except the nematodes). As previously reported, *miR-993* belongs to the *miR-100/10* family, and both *miR-993* and *miR-10* are derived from the ancient *miR-100* through duplication and arm-switching [51]. The higher expression of *miR-993* (*miR-993-3p*) compared with *miR-993** (*miR-993-5p*) was found in *Drosophila melanogaster* [52]. However, in many other insects, including *Tribolium castaneum* and *B. mori*, *miR-993**s are expressed at higher levels than *miR-993*s [46, 53]. Therefore, the relative expression of mature miRNAs in the opposite arms of one precursor differs from species to species. Arm-switching allows some miR*s to become functional miRNAs. Because the *miR-100/10* family members are located within *Hox* complexes and can regulate the expression of the relevant *Hox* genes [54], and *Hox* genes are involved in development regulation, *miR-993*s may play a role in insect development by targeting *Hox* genes.

Most identified miRNAs are insect-specific

Most of the miRNAs identified in this study

were insect-specific. As there are many insect-specific features, such as metamorphosis and pheromones [55], these miRNAs may specifically regulate insect genes and relevant pathways. A typical example is *miR-14*, which is found only in insects. Previous studies have demonstrated its involvement in a positive autoregulatory loop controlling ecdysone signaling in Drosophila [16], which plays a key role in metamorphosis. We believe the other insect-specific miRNAs may also be involved in insect-specific features. Because insects constitute the most diverse group of animals, studies on these miRNAs can provide cues on insects' extensive adaptivity.

Among insect-specific miRNAs, 29 har-miRNAs and 20 sli-miRNAs were regarded as Lepidoptera-specific (Supplementary Material: Tables S4 and S5), and we hypothesize that they have specific functions in lepidopteran insects and could possibly be used as targets for lepidopteran pest control. In both species, nine Lepidoptera-specific miRNAs, including *miR-9c, miR-92b, miR-306a, miR-308, miR-745, miR-2755, miR-2766, miR-2767* and *miR-2768*, have homologs in all the 6 analyzed lepidopteran insects, indicating that they have conserved functions in lepidopteran species. Another Lepidoptera-specific miRNA, *miR-1175-5p*, has homologs in only the 3 types of moths, but not in butterflies, and thus it may have specific functions in moths, most likely regulating moth-specific genes. Although we cannot be sure of their inexistence in other species due to the absence of genomic information for most insects, particular attention should be paid to these miRNAs with respect to their specific functions in lepidopteran insects or in moths. These miRNAs may be involved in some special biological processes that distinguish Lepidoptera (or moths) from other species.

Some conserved miRNAs are involved in Lepidoptera development

The developmental expression profiles of the miRNAs *miR-2a*, *miR-34*, *miR-2796-3p* and *miR-11* were investigated by qRT-PCR. These miRNAs showed different expression patterns (Fig. 4). According to the rationale that the high expression of an miRNA at a specific stage suggests its possible regulatory role in that stage [56], the different patterns of the miRNAs indicate their diverse functions during development. The high expression of *miR-2a* and *miR-11* in pupae reflects their possible involvement in metamorphosis, whereas the high level of *miR-34* and *miR-2796-3p* in the adult stage may indicate their roles in adult development or reproduction. In a comparison with the expression of relevant miRNAs in *B. mori* reported by Zhang et al [57], *miR-34* showed a higher expression in the adult stage in all three species, indicating its important and possibly conserved role in adult development. Interestingly, the low expression of *miR-34* in pupae is similar to that observed in Drosophila, in which *miR-34* was found to be under the control of two key hormones regulating metamorphosis. In detail, Drosophila *miR-34* is repressed by ecdysone and induced by juvenile hormone [15]. Thus, we infer that the hormonal regulation of *miR-34* and its involvement in metamorphosis may be conserved in these insects. However, *miR-2a*, which was called *miR-2* by Zhang et al [57], was expressed at the highest levels at the embryonic and adult stages of *B. mori* but at the pupal stage in *H. armigera* and *S. litura*, indicating that some conserved miRNAs likely have distinct functions in different species.

The conserved regions in the 3' UTRs of *B. mori*, *H. armigera*, and *S. litura* genes implied that they may have conserved miRNA target sites. Through the target prediction of homologous regions in the 3' UTRs, we identified a number of genes that could potentially be targeted by common miRNAs (Table 3). These targeted genes included transcription factors, heat shock proteins, and genes involved in hormone pathways or metabolism. The diverse genes indicate the various roles miRNAs can play in different species. However, most of the genes play a role in development. For example, genes involved in hormone pathways, such as *EcR*, *USP* and *PBANR*, are related to metamorphosis. Metabolism is also related to insect growth because aberrant metabolism may lead to abnormal development. Transcription factors are regulators of most genes involved in all types of biological processes, including development. Moreover, heat shock proteins are involved in stress adaptation and development. Therefore, although the conserved miRNAs may play a role in diverse biological processes, they affirmably participate in the regulation of development.

Among these genes, *Hsp90* was selected to validate its regulation by the miRNAs because its 3' UTR was predicted to contain 3 conserved miRNA target sites. HSP90 is a conserved protein playing a role not only in development, but also in cancer progression, evolutionary diversification, and the regulation of coding and non-coding genes including miRNAs [58]. We used a dual-luciferase reporter assay for the target validation because this is a commonly used method to visualize miRNA-dependent gene silencing [59]. According to our preliminary experiments, lepidopteran cell lines showed low transfection efficiency, which impaired the down-regulation of reporters by the corresponding miRNAs (data not shown) and may lead to the neglect of some weak effects of miRNAs on

gene regulation. Therefore, the mammalian 293T cell line was used instead for this assay because of its high transfection efficiency. According to the results (Fig. 5B), the 3 miRNA target sites in the *Hsp90* 3' UTRs were proven to be authentic. The results indicated that conserved genes in different insect species could be targeted by common miRNAs and that such conserved regulation may play roles in various biological processes. The regulation of *Hsp90* by miRNAs indicates that these conserved miRNAs are most likely involved in the regulation of development, and that miRNAs may regulate coding and non-coding genes through *Hsp* genes.

Summary

In summary, this study provides a new method for miRNA identification in non-model insects lacking genome information. Moreover, the analysis of expression patterns and the validation of conserved miRNA targets revealed the diversity and conservation of miRNA functions in different insect species. The results extend our understanding of insect miRNA biological functions and will shed light on the identification of insect-specific miRNA targets that can be used in the future for lepidopteran pest control.

Supplementary Material

Table S1~S5, and Fig.S1.
http://www.biolsci.org/v09p0001s1.pdf

Abbreviations

miRNA: microRNA; nt: nucleotides; RISC: RNA-induced silencing complex; miR*: miRNA-star strand; PCR: polymerase chain reaction; qRT-PCR: quantitative reverse transcription polymerase chain reaction; sRNA: small RNA; SBS: sequencing-by-synthesis; UTR: untranslated region; har-miRNA: *Helicoverpa armigera* miRNA; sli-miRNA: *Spodoptera litura* miRNA.

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Competing Interests

The authors have declared that no competing interest exists.

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