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Computational prediction of microRNA genes in silkworm genome*

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Abstract: MicroRNAs (miRNAs) constitute a novel, extensive class of small RNAs (~21 nucleotides), and play important gene-regulation roles during growth and development in various organisms. Here we conducted a homology search to identify homologs of previously validated miRNAs from silkworm genome. We identified 24 potential miRNA genes, and gave each of them a name according to the common criteria. Interestingly, we found that a great number of newly identified miRNAs were conserved in silkworm and *Drosophila*, and family alignment revealed that miRNA families might possess single nucleotide polymorphisms. miRNA gene clusters and possible functions of complement miRNA pairs are discussed.

Key words: miRNA, Silkworm, Computational prediction
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INTRODUCTION

MicroRNAs (miRNAs) represent a class of noncoding small RNAs (~22 nucleotides) identified from various organisms extending from nematodes to humans (Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Lee and Ambros, 2001). They derive from pre-miRNAs extracted from primary transcripts (pri-miRNA) and can form stable hairpin structures. Pre-miRNAs are processed by RNase III enzyme Dicer, which also cleaves long, perfectly double-stranded RNA into 21~22 nucleotides (nt) siRNA duplexes (Hannon, 2002).

There are two types of mechanisms for miRNA-dependent regulation of gene expression: (1) target mRNAs which can perfectly complement miRNAs cleaved by RISC (RNA-induced silencing complex); (2) miRNAs which inhibit the expression of mRNAs that are imperfectly complementary to miRNA. Functions of several miRNAs have been reported before, although biological functions of miRNAs are not yet completely understood. The founding members of miRNAs are lin-4 and let-7 in *C. elegans* (Lee *et al*., 1993; Reinhart *et al*., 2000), which are also called small temporal RNAs because their inactivative mutation affects developmental timing (Banerjee and Slack, 2002). Lin-4 and let-7 exerted their functions by complementing to 3′ untranslated region (3′ UTR) of target mRNAs and inhibiting their translation processes (Lee *et al*., 1993; Reinhart *et al*., 2002). In *Drosophila*, the defect of miR-14 leads to reaper-dependent cell death and increases levels of triacylglycerol and diacylglycerol, which are required for fat metabolism (Xu *et al*., 2003); miRNA encoded in *bantam* locus controls the cell proliferation and regulates the proapoptotic gene *hid* (Brennecke *et al*., 2003). These findings raise the possibility that miRNAs perform as negative regulators to control cell proliferation, differentiation, and other developmental processes. Similar to animal miRNAs, plant miRNAs exhibit temporal and tissue-specific expression patterns (Llave *et al*., 2002; Mourelatos *et al*., 2002; Park *et al*., 2002; Reinhart and Bartel, 2002).

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Many target genes which perfectly complement plant miRNAs were found, however, identification of putative miRNAs targets in animals is much more complex, because imperfect complementarities between miRNAs and target genes increase the difficulties in investigating the interaction between miRNAs and target mRNAs.

Up to now, hundreds of miRNAs, usually having multiple copies in vivo, have been identified from various species. For instance, in nematodes, most identified miRNAs present very high steady-state levels—more than 1000 molecules per cell, with some exceeding 50000 molecules per cell (Lim *et al*., 2003). Llave *et al*.(2002) identified 125 small RNAs between 16 and 25 nt; only four of these met the criteria (Ambros *et al*., 2003) of miRNAs. Similarly, Park *et al*.(2002) identified 230 unique sequences, five of which appeared to be miRNAs. Nowadays, a great proportion of known miRNAs were identified by computational approaches or by the combination of biochemical methods and computational procedures. It seems that few miRNAs have not been uncovered in model organisms. On the other hand, miRNAs from non-model organisms, such as silkworm, have not yet been reported.

Recently, draft sequence of domesticated silkworm genome has been accomplished. It revealed that there are 18510 genes in the silkworm genome, which far exceeds the official gene count of 13379 for fruitfly and that a great number of silkworm genes have homologs in fruitfly (Xia *et al*., 2004). These accomplishments made it possible to investigate function genes in silkworm genome by implementing computational procedures. In the present study, we conducted a homology search to select new silkworm miRNA genes that are homologous with identified miRNAs from other organisms. As a result, we identified many paralogs of known miRNAs with one or more different nucleotides with most potential miRNAs perfectly complementing with query sequences from *Drosophila*.

MATERIALS AND METHODS

Identified mature miRNAs of model organisms (Mar. 2005, downloaded from http://166.111.30.65/micrornadb/index.php) were used as query sequences to BLAST against silkworm genome (http://www.dna.affrc.go.jp/database/silkworm.html) with default parameters and a non-stringent cutoff of E>1.8 (Lim *et al*., 2003). Then we picked out flanking sequences of hit sites (about ±60 nt) from silkworm genome sequences. The extracted sequences were submitted to Mfold Web server (Zuker, 2003) to predict fold-back structures. Sequences were eliminated either more than 3 internal nucleotides mismatched to query sequences or total mismatched nucleotides of over 4 nt, or the free energy of the second structures were higher than -1.05×10^2 kJ/mol (Lim *et al*., 2003).

For further validation, all identified sequences that can form stable stem-loop structures were aligned with their family members and subjected to phylogenetic analysis on the relationship between members in each family by using ClustalX and PHYLIP (download from http://evolution.genetics.washington.edu/phylip.html).

RESULTS

Identification of 24 miRNA genes from silkworm genome

In the present research, we adopted a homology search procedure to search for miRNA genes that are homologous with identified miRNAs from model organisms. As a result, 24 novel potential miRNA genes were identified from silkworm genome (Table 1). With each of them given a name according to the common criteria such as bmo-let-7 (*Bombyx mori*, bmo-). Twenty-two newly identified sequences were homologous with query miRNAs from *Drosophila* and two had *C. briggsae* homologs. We failed to find any sequences matching query miRNAs from the other species, excepting that seven sequences (bmo-let-7, bmo-mir-1, bmo-mir-7, bmo-mir-8, bmo-mir-9a, bmo-mir-124, and bmo-mir-263b) had homologs in several species in addition to *Drosophila*.

Most identified miRNA genes perfectly matched the query sequences, while the remaining were homologous with query sequences with one or more mismatching nucleotides. Imperfectly matched miRNA genes either had different nucleotides at their 5′/3′ terminus compared to query sequences or contained diverged nucleotides in their internal regions. In total, the number of mismatched nucleotides in

Table 1 Candidate microRNAs in silkworm genome

Name[a]	Source[b]	Match extend[c]	Matched sequence[d]	Fold-back size[e]
bmo-let-7	AADK01002497 (10858-10878)	21/21	TGAGGTAGTAGGTTGTATAGT	96
bmo-miR-1	AADK01005296 (6087-6108)	22/22	TGGAATGTAAAGAAGTATGGAG	97
bmo-miR-7	AADK01002696 (14044-14066)	23/23	TGGAAGACTAGTGATTTTGTTGT	98
bmo-miR-8	AADK01023997 (815-837)	23/23	TAATACTGTCAGGTAAAGATGTC	98
bmo-miR-9a	Contig551684 (34-56)	23/23	TCTTTGGTTATCTAGCTGTATGA	88
bmo-miR-10c	AADK01012541 (8182-8201)	17/21	CTCTCTAGAACCGAATTTGT	96
bmo-miR-14	AADK01001451 (17523-17543)	21/21	TCAGTCTTTTTCTCTCTCCTA	96
bmo-miR-31c	AADK01000344 (33951-33971)	20/23	GGCAAGAAGTCGGCATAGCTG	98
bmo-miR-34	Contig567840 (1436-1457)	22/22	TGGCAGTGTGGTTAGCTGGTTG	97
bmo-miR-71	AADK01011192 (8467-8485)	19/19	TGAAAGACATGGGTAGTGA	64
bmo-miR-124	AADK01010415 (7041-7063)	23/23	TAAGGCACGCGGTGAATGCCAAG	98
bmo-miR-263b	AADK01025663 (3376-3396)	21/21	CTTGGCACTGGGAGAATTCAC	96
bmo-miR-263c	Contig476700 (2833-2853)	21/24	AATGGCACTGGAAGAATTCAC	99
bmo-miR-275	AADK01007727 (11262-11284)	23/23	TCAGGTACCTGAAGTAGCGCGCG	70
bmo-miR-276b	AADK01021256 (906-927)	22/23	AGCGAGGTATAGAGTTCCTACG	97
bmo-miR-276a*	AADK01021256 (941-962)	22/22	TAGGAACTTCATACCGTGCTCT	97
bmo-miR-277	Contig132079 (12099-12121)	23/23	TAAATGCACTATCTGGTACGACA	108
bmo-miR-279b	Contig467689 (714-732)	19/22	TGACTAGATCCACACTCAT	87
bmo-miR-282b	AADK01024756 (1421-1445)	24/28	ACCTAGCCTCTCCTTGGCTTTGTCTGT	87
bmo-miR-283	AADK01009773 (8152-8172)	21/21	TAAATATCAGCTGGTAATTCT	96
bmo-miR-305	AADK01007727 (11352-11374)	23/23	ATTGTACTTCATCAGGTGCTCTG	63
bmo-miR-307	AADK01002680 (19093-19112)	20/20	TCACAACCTCCTTGAGTGAG	95
bmo-miR-iab-4	AADK01004318 (3477-3498)	22/22	ACGTATACTGAATGTATCCTGA	97
bmo-miR-iab-4*	AADK01004318 (3515-3538)	24/24	CGGTATACCTTCAGTATACGTAAC	97

[a]Each miRNA gene is given a name according to common convention; [b]Silkworm genome sequences that contain novel miRNA genes; [c]The extent of the match is indicated in the format of "number of matched ncleotides/query sequence length"; [d]Matched sequences in silkworm genome, and mismatched nucleotides are indicated as little characters; [e]Predicted length of identified miRNAs precursors; * means the miRNA is produced from antisence strand

potential miRNA genes was less than 4 nt, providing they had the same length as their query sequences. Although these sequences extended from 19 to 27 nt, they had a much tighter distribution, centering on 21~24 nt which were coincident with known specificity of Dicer processing. Interestingly, most identified sequences had composition preferable beginning with an uridine at the 5′ terminus, which also had been observed in miRNAs from other organisms (Lee and Ambros, 2001; Lagos-Quintana *et al*., 2002; Reinhart *et al*., 2002). Twenty-four potential miRNAs can be grouped into 22 families, each comprising of 2 to 28 genes. Within these families, consensual sequences either span through the whole length of miRNAs or are predominantly at their 5′/3′ terminus.

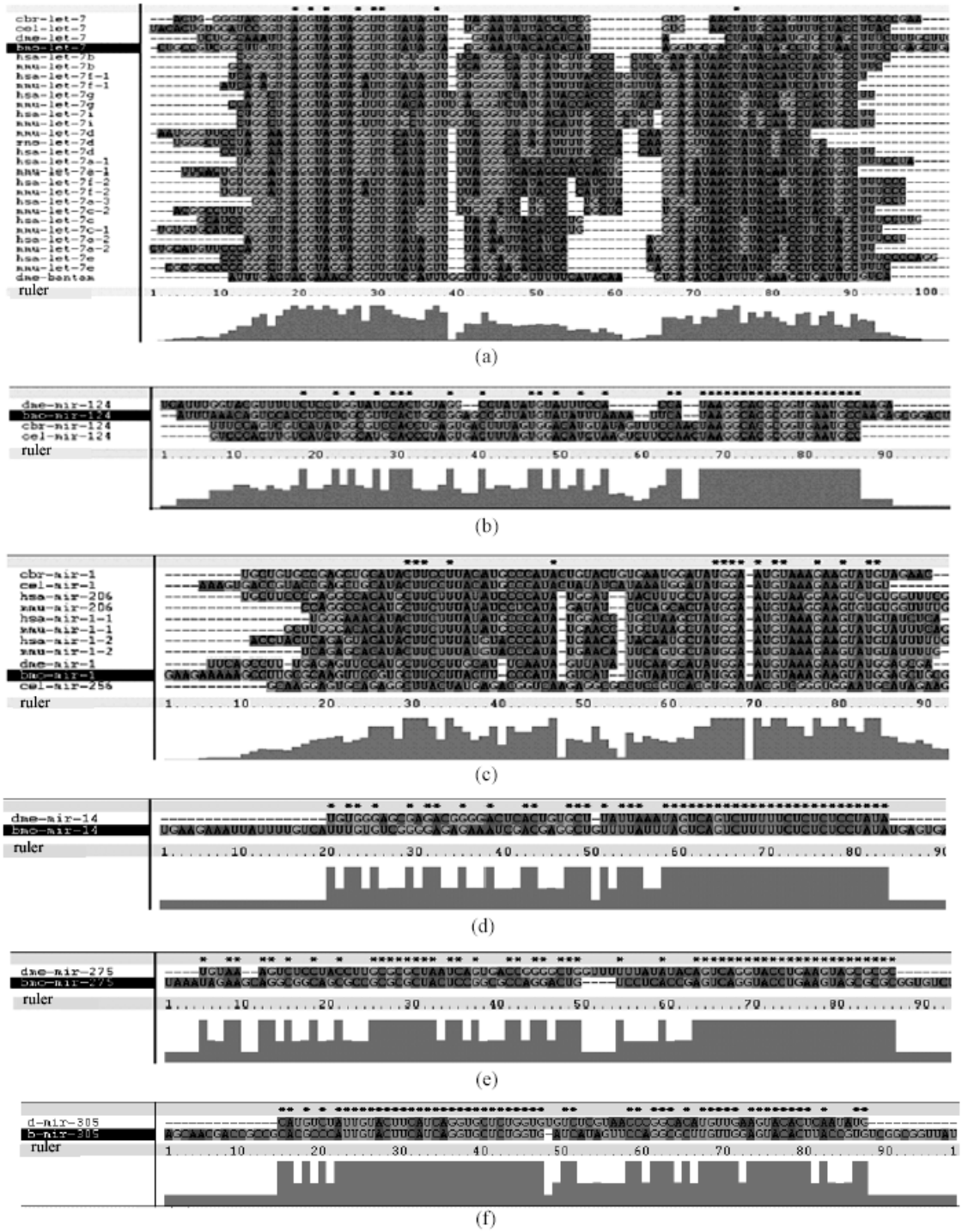
Of the 22 families, 6 largest families contained over 10 miRNAs while 9 families only had 2 members from *Drosophila* and silkworm.

Homology analysis

According to the criteria of miRNA annotation, homologs of previously validated miRNAs need not meet as stringent criteria to be annotated as additional miRNA *loci*. Very close homologs in other species can be annotated as miRNA homologs without experimental validation, if they have phylogenic conservation and predicted fold-back precursor secondary structure (Ambros *et al*., 2003). In order to furher verify candidate miRNAs, pre-miRNAs from each family were subjected to family alignment process using Clustal X, including known miRNAs and novel candidates.

Although let-7 family contained more than 30 members, extending from worm through human, both miRNA-encoding regions and complementary sequences showed high conservation (Fig.1). Mir-124 family, mir-276 family and mir-71 family showed

dramatically high conservation that over 20 continuous nucleotides were identical. Four families (mir-307, mir-7, mir-263b and mir-8) contained over 10 continuously identical nucleotides within miRNA-coding regions. However, both flanking regions and loops are variable (Fig.1). Interestingly, alignment results showed that most families might have characteristics of single nucleotide polymorphism.



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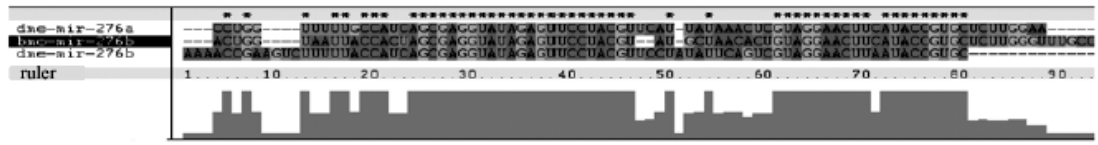

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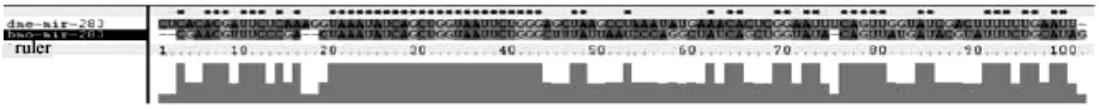

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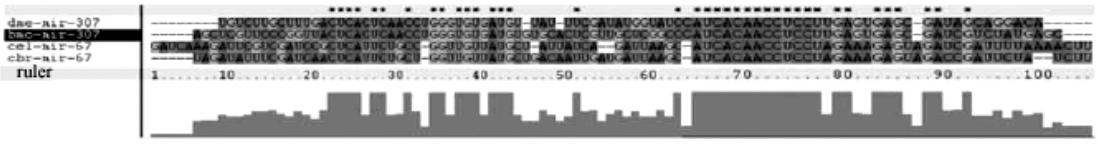

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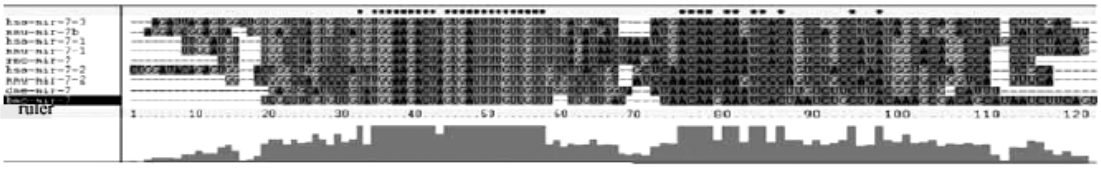

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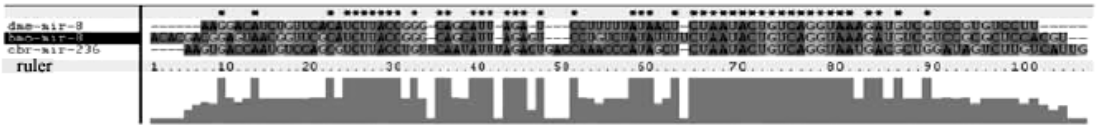

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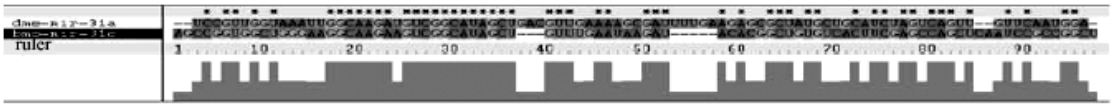

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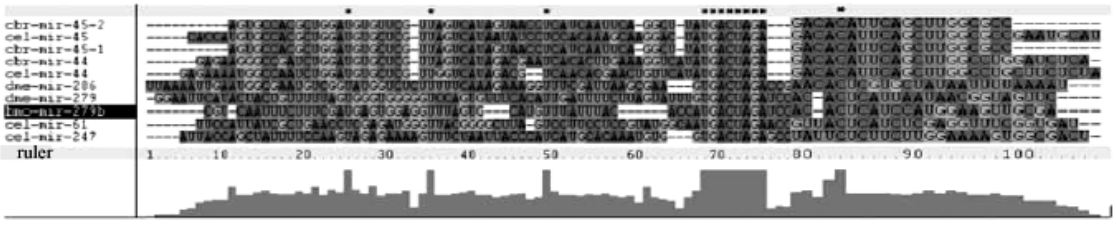

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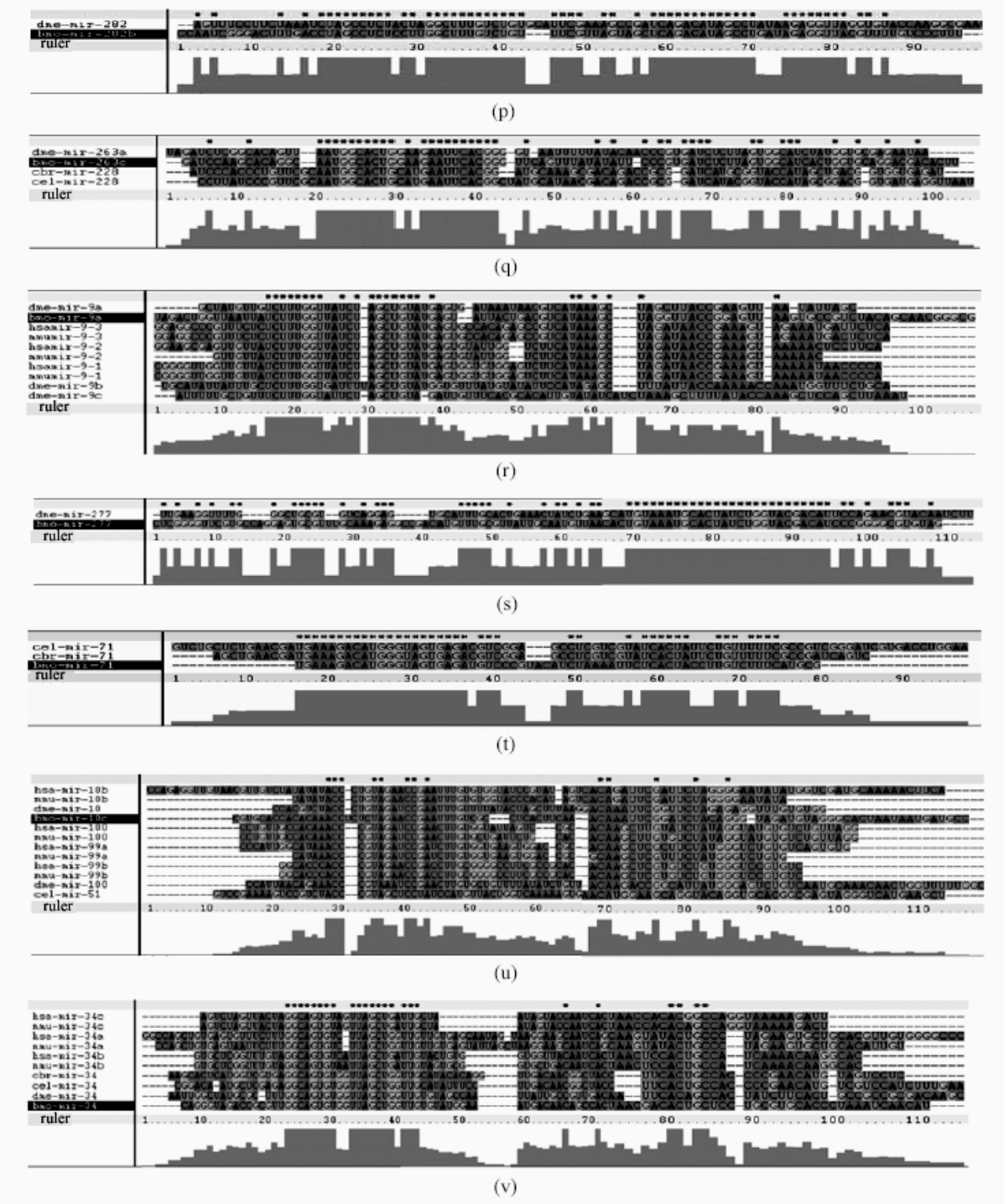


Fig.1 Sequence alignments of pre-miRNAs in each miRNA family. (a) let-7 family; (b) mir-124 family; (c) mir-1 family; (d) mir-14 family; (e) mir-275 family; (f) mir-305 family; (g) mir-276 family; (h) mir-263b family; (i) mir-283 family; (j) mir-307 family; (k) mir-7 family; (l) mir-8 family; (m) mir-iab-4 family; (n) mir-31c family; (o) mir-279 family; (p) mir-282 family; (q) mir-263a family; (r) mir-9a family; (s) mir-277 family; (t) mir-71 family; (u) mir-10 family; (v) mir-34 family

Members of each miRNA family that contain novel silkworm miRNAs are subjected to sequence alignment; Precursors of silkworm miRNAs are indicated by black ground. The length of pre-miRNAs consistent with that of foldback predicted sequence. Dark dot in the first line of pictures indicates constant nucleotide

Phylogeny analysis

Pre-miRNAs from six largest families were subjected to phylogeny analysis using dnapairs program in PHYLIP package for analyzing revolution relationship of members from each family. Most silkworm miRNAs were located together in branches with related *Drosophila* miRNAs, with the exception of bmo-mir-10c, which was relatively less related to any *Drosophila* microRNA (Fig.2). Commonly, human miRNAs had close revolution relationship with mouse miRNAs (Fig.2). It might attribute to the fact that a number of miRNAs in the two species share identical sequences. Let-7 family contained more than 30 sequences from divergent species, with most of them from mammalians (Fig.1). Interestingly, both m-let-7c-1 and h-let-7c located at shortest branches of let-7 family tree (Fig.2), indicating they may be the earliest members of let-7 family.

Clusters and complement miRNA pairs

Mapping miRNA genes into silkworm genome, we discovered bmo-miR-275 and bmo-miR-305 were hosted in single sequence as their homology from *Drosophila*. However, the distance between these two silkworm miRNA genes was farther. As similar phenomenon had been reported before, we predicted that there might be more clusters existing in silkworm. Indeed, applying similar strategy, we found another two clusters (bmo-miR-iab-4/bmo-miR-iab-4* and bmo-miR-276b/bmo-miR-276a*). Moreover, bmo-

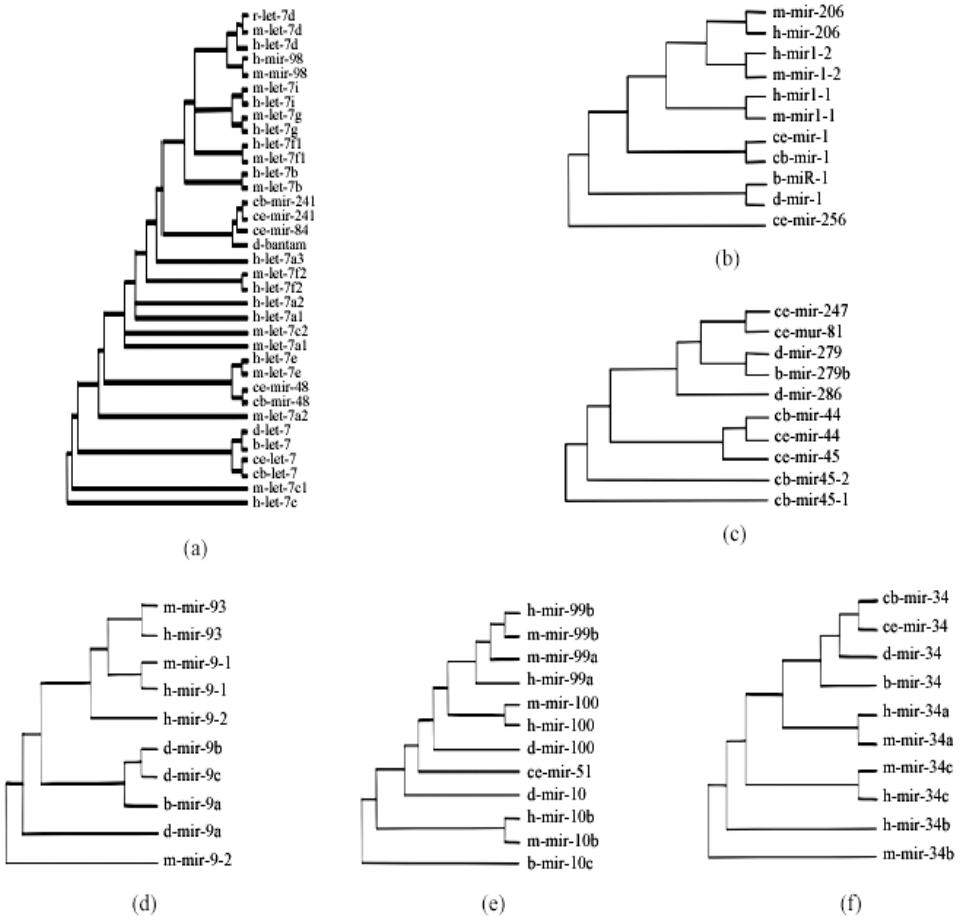


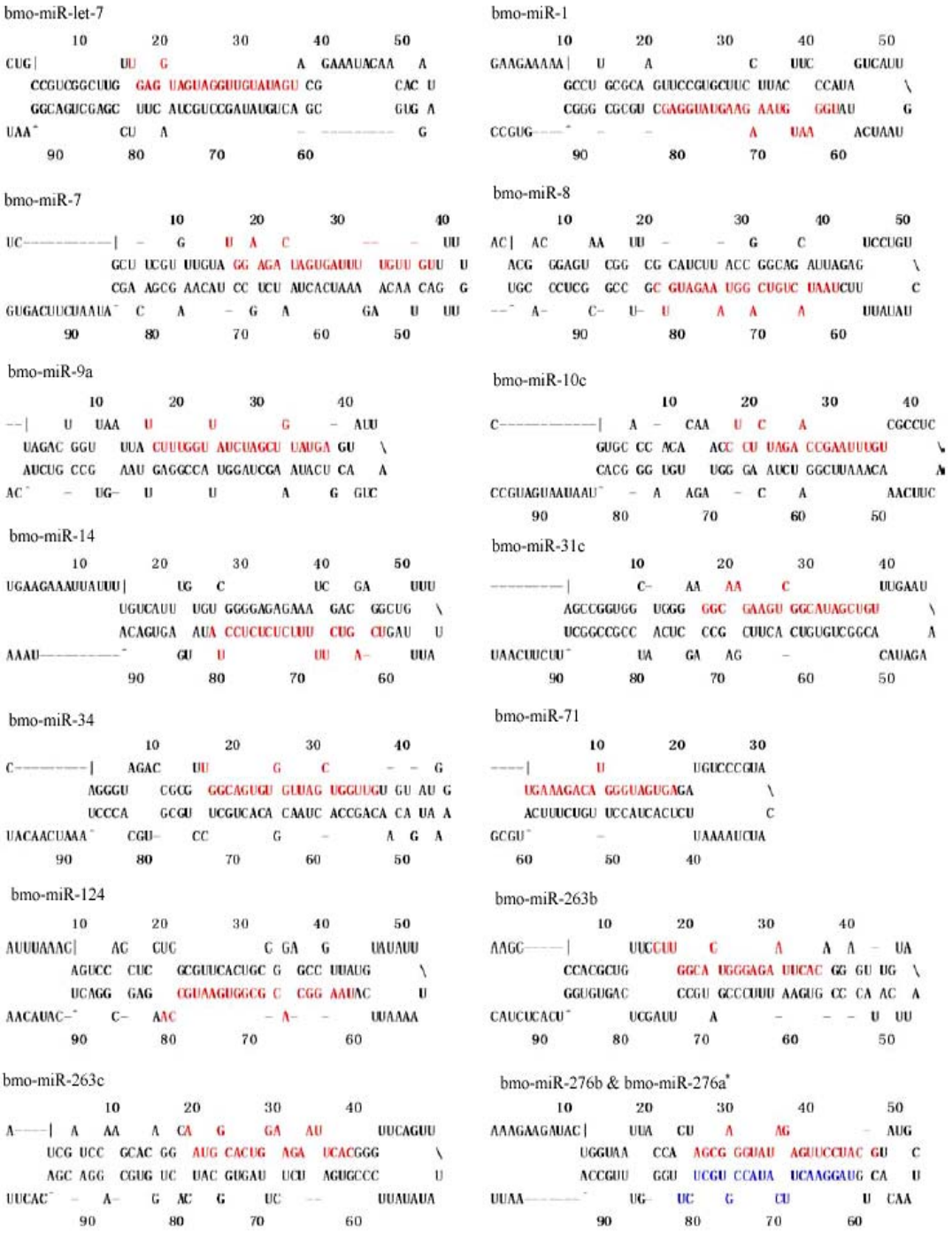
Fig.2 Phylogeny analysis of six large families. (a) let-7 family; (b) mir-1 family; (c) mir-279 family; (d) mir-9 family; (e) mir-10 family; (f) mir-34 family

Members from six large families, which contain novel miRNAs in the present study, were submitted to construct phylogeny trees in order to analysis the relationship between family members from different species. Abbreviations are indicated as follows: h: human; r: *Rattus norvegicus*; m: *Mus musculus*; d: *Drosophila melanogaster*; b: *Bombyx mori*; cb: *Caenorhabditis briggsae*; ce: *Caenorhabditis elegans*

miR-iab-4/bmo-miR-iab-4* and bmo-miR-276b/bmo-miR-276a* can complement with each other, respectively (Fig.3). Fold-back prediction showed these four miRNAs came from two miRNA precursors.

DISCUSSION

Silkworm is a model organism of lepidoptera that is widely used in industry, especially in textile production. Up to now, hundreds of miRNAs have



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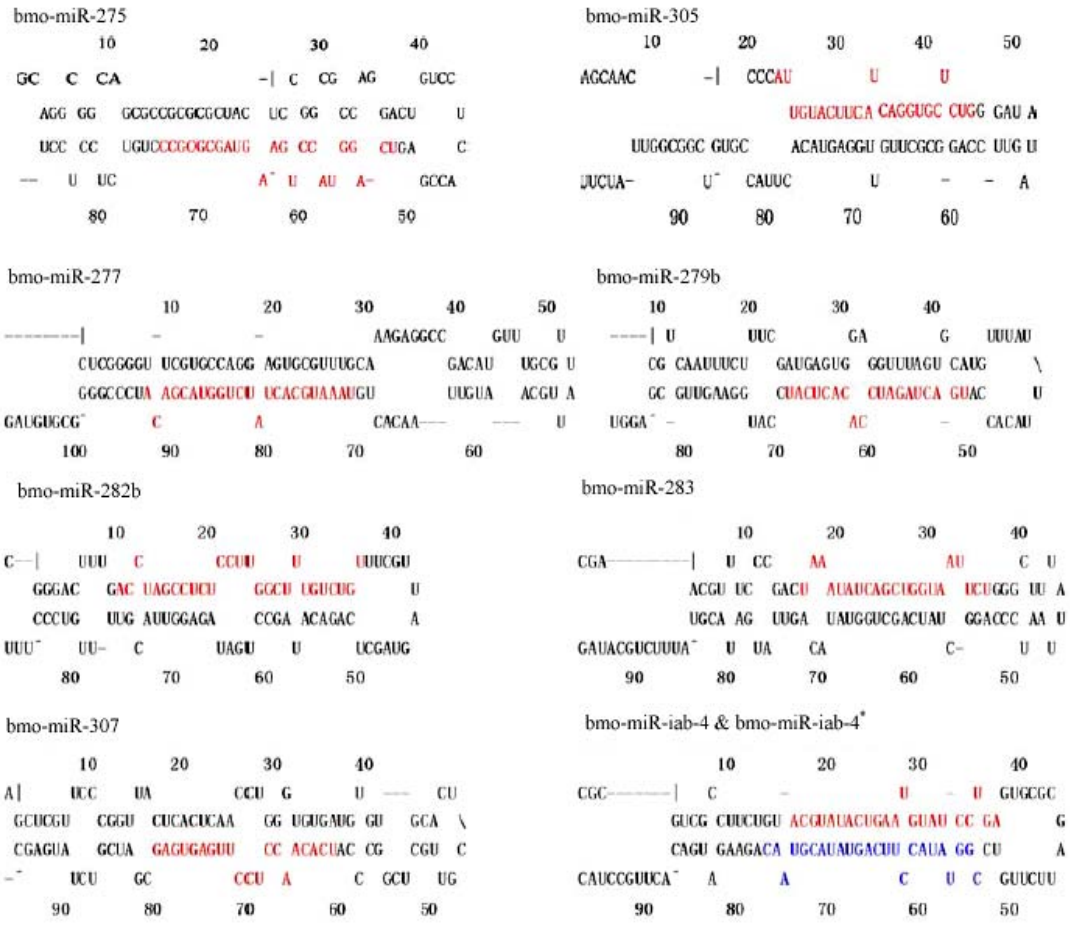

Fig.3 Predictions of secondary structures of novel miRNAs from silkworm genome

Each of novel miRNAs is submitted to Mfold Web server to verify whether they can form fold back structure and whether their secondary structure is stable or not. Mature miRNA indicated by red or blue. miRNAs produced from antisense strand of pre-miRNA are indicated as blue characters

been identified from various species; although, miRNAs from silkworm have not yet been reported. In the present study, by applying homologous alignment procedure, we found 24 potential miRNA genes located in various sites of the silkworm genome. Most of the novel miRNA genes were highly homologous to query miRNAs from other species and the fold-back prediction showed each of the potential miRNAs located in either arm of hairpin structure, which is consistent with query miRNAs. Moreover, sequences extracted from flanking regions of hit *loci* can form stable stem-loop structure ($dG \leq -1.05\times10^2$ kJ/mol). Based on these observations and the annotation criteria for homology of previously validated miRNAs (Ambros *et al*., 2003), we proposed that these *loci* could be annotated as miRNA genes. Of course, we cannot exclude the possibility that they are pseudo-miRNAs due to lack of direct experimental evidence.

Phylogeny analysis revealed that within six largest miRNA families in the present research, human miRNAs usually had closer revolution relationship with mouse miRNAs than the other species (Fig.2). This phenomenon might be due to the miRNAs characteristic of high conservation, as organisms that have close phylogeny relationship usually share a number of miRNAs, although their pre-miRNAs might be dramatically different.

Besides, single nucleotide polymorphism (SNP) patterns were obvious for most of the miRNA families, especially in the conservation regions that produce matured miRNAs. In mammalians, SNPs are

important for discriminating different species because they usually have different SNP contribution patterns. So we proposed that SNPs might also represent the differentiation of miRNAs from various species. As most SNPs reside in conserved regions encoding mature miRNAs, changes in these sites may influence the interaction between Dicer and miRNA precursors, and then causing asymmetric or symmetric cleavage. In animals and plants, the mechanisms of miRNA-dependent regulation were different; changes in some sites of mature miRNAs might also direct the way of gene regulation. These hypotheses need further experiments to verify, as characteristics of SNP in miRNAs have not yet been reported.

Mapping miRNA genes onto genome, revealed that each of three miRNAs pairs (bmo-mir-275/bmo-mir-305, bmo-miR-iab-4/bmo-miR-iab-4* and bmo-miR-276b/bmo-miR-276a*) is clustered into single sequence as their query miRNAs, although internal length between two silkworm miRNAs is longer. Phenomenon like such cases had already been reported from various species (Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Mourelatos *et al*., 2002). In fact, the clustering propensity of miRNA genes was noticed from the early days of the massive direct cloning of short ncRNA molecules (Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Mourelatos *et al*., 2002; Aravin *et al*., 2003). Usually, there are two or three miRNA genes in a cluster, although larger clusters were also identified, such as h-miR-17 cluster composed of six members (Lagos-Quintana *et al*., 2001; Mourelatos *et al*., 2002), which is also conserved in other mammals (Tanzer and Stadler, 2004), or the *Drosophila melanogaster* cluster of eight miRNA genes (Aravin *et al*., 2003). Recently, Seitz *et al*.(2004) predicted a huge cluster of 40 miRNA genes located in the ~1 Mb human imprinted 14q32 domain, several of which were shown to be expressed. Clustered miRNA genes might show high similarity in nucleotide composition, but sometimes they could also differ (Aravin *et al*., 2003; Bartel, 2004). The expression profiles of clustered genes are highly similar, raising the possibility that transcription of such miRNAs is controlled by common regulatory elements (Lee *et al*., 2002). It was supposed that a single promoter caused transcription of the clustered miRNAs genes (Lee *et al*., 2002). If so, these clustered genes might simultaneously participate in a common regulation pathway.

Recent research showed that pre-miRNAs are transported to the cytoplasm, where they are recognized by the RISC containing Dicer-TRBP-Ago2. The RNase III Dicer cleaves ~22 nt from the Drosha cleavage site, thus generating ~22 nt duplex miRNA (2 nt 3′ overhangs), which remained associated with RISC as a ribonuleoprotein complex. The complex identified the guide strand of the RNA duplex, perhaps through TRBP's and Dicer's recognizing thermodynamic asymmetry between the two ends of the duplex. Then miRNAs* are degraded, leaving miRNA which still resided in RISC (Gregory *et al*., 2005). At a dramatically low rate, both arms of the hairpins could give rise to miRNAs (Lim *et al*., 2003) exemplified by bmo-miR-iab-4:bmo-miR-iab-4* and bmo-miR-276b:bmo-miR-276a* (Fig.3). miRNA* recovered from duplex RNA may be due to the duplex RNA lack of structural features enforcing asymmetric RISC assembly (Schwarz *et al*., 2003). We noticed the possibility that antisense miRNAs play important roles in negatively regulating miRNAs function, and even regulating gene expression independently as exemplified by miR-131 (miR-9*), which is a vertebrate Brd-box family miRNA and potential homolog of miR-79 (Lai *et al*., 2004). In all, as more details on the function of miRNAs are being revealed, we will understand better these complementary miRNA pairs.

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