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Full Length Research Paper

Bioinformatic identification of microRNAs and their targets in *Aquilegia formosa x Aquilegia pubescens*

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As a model system, *Aquilegia* is of evolutionary and ecological significance. Availability of new genomic resources is facilitating the related researches at molecular level. MicroRNAs (miRNAs) are a class of endogenous, non-coding and short RNAs directly involved in regulating gene expression at the post-transcriptional level. High conservation of miRNAs in plants provides the foundation for identification of conserved miRNAs in other plant species through homology alignment. For the purpose of finding miRNAs in Aquilegia Formosa x *Aquilegia pubescens,* previous known plant miRNAs were, plant were used for BLAST search against its expressed sequence tag (EST) database and following a series of filtering criteria, 12 new miRNAs belonging to 5 miRNA families were identified while 51 potential target genes were subsequently predicted, most of which seemed to encode transcription factors or enzymes participating in regulation of development, growth, metabolism and other physiological processes. These findings not only lay the foundation for understanding the roles of miRNAs in *Aquilegia,* but also provide a phylogenetically important dataset for plant miRNA evolution studies.

Key words: *Aquilegia*, bioinformatic analysis, microRNA, evolution.

INTRODUCTION

The lower eudicot genus *Aquilegia* represents a phylogenetic midpoint between the eudicot and monocot models such as *Arabidopsis* and *Oryza*, and holds enormous potential for investigating aspects of development, ecology and evolution (Kramer, 2009). Besides, species in this flowering plant genus have undergone a very recent adaptive radiation and present a unique opportunity to investigate the molecular genetic changes underlying adaptations (Kramer, 2009; Puzey and Kramer, 2009).

Aquilegia formosa and *Aquilegia pubescens* are two closely related species belonging to the columbine genus (Cooper et al., 2010). Despite their morphological and ecological differences, hybrid population can form when hybrid zone is established. Though, the importance of hybridization in adaptive radiation and evolution has been debated for decades, recent molecular genetic studies have indicated that hybridization is surprisingly frequent in natural populations, which can lead to rapid genomic changes, including chromosomal rearrangements, genome expansion, different gene expression and silencing and the beneficial new phenotypes (Rieseberg, 2009). Availability of genomic data will produce a new understanding of the genetic nature of species and will help resolve a century-old debate over the role of hybridization (Baack and Rieseberg, 2007).

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding and single-stranded RNAs that act as post-transcriptional regulators in eukaryotes (Unver et al., 2009). They can control many important aspects of plant development, suggesting that these molecules may also have played key roles in the evolution of developmental processes in plants (Jasinski et al., 2010). In recent years, the evolution and conservation of plant miRNAs has been the subject of significant investigation (Axtell and Bowman, 2008). Although the roles of miRNAs have been extensively studied, their expression diversity and

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Abbreviations: miRNAs, MicroRNAs; **ESTs,** expressed sequence tags.

evolution in closely related species and interspecific hybrids are poorly understood (Ha et al., 2009).

In *Aquilegia*, miRNAs only have been reported in *Aquilegia coerulea* (Puzey and Kramer, 2009). Availability of new genetic and genomic resources, especially the publishing of EST database of *A. formosa x A. pubescens* (http://www.ncbi.nlm.nih.gov/) provides the chance to investigate the expression diversity and evolution in these closely related species and interspecific hybrids. Nowadays, two major categories of approaches have been applied for miRNAs investigation (Unver et al., 2009). Compared to the experimental approaches, computational methods have been proved to be faster, more affordable and more effective, contributing mostly to today's plentiful storage in miRBase (Unver et al., 2009). Different computational miRNA finding strategies have been developed based on a core principle of looking for conserved sequences among different species that can fold into extended hairpins (Bonnet et al., 2004). The biogenesis of miRNAs suggests that it is possible to find miRNAs by searching expressed sequence tags (ESTs) with known miRNAs. There have been more and more reports about the identification of miRNAs by mining the repository of available ESTs (Lu and Liu, 2010a; Han et al., 2009; Zhang et al., 2009; Song et al., 2009; He et al., 2008; Xie et al., 2007; Zhang et al., 2008). EST analysis makes it possible to rapidly study miRNAs and their functions in species whose genome sequences have not been well known (Zhang et al., 2006a).

The goal of this study is to identify new miRNAs from the EST sequences of *A. formosa x A. pubescens* through bioinformatic analysis. The findings will lay foundation for further research of the roles of miRNAs in *Aquilegia* and also will provide a phylogenetically important dataset for plant microRNA evolution studies.

MATERIALS AND METHODS

Sequences and softwares

The known plant miRNA sequences from *Arabidopsis*, *Brassica*, *Glycine*, *Saccharum*, *Sorghum*, *Vitis*, *Solanum*, *Oryza*, *Triticum*, *Chlamydomonas* and other plant species were downloaded from the miRNA database miRBase (http://www.mirbase.org) (Release 14: September 2009). After removal of the repeated sequences, 2177 items were left as the reference set. The 85041 EST sequences of *A. formosa x A. pubescens* and 12313 GSS sequences of *A. formosa* and *Aquilegia vulgaris* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), Blast-2.2.21-ia3 was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/Ftp/) and set up locally. RNA secondary structure and the free energy were calculated by web server mfold (http://mfold.bioinfo.rpi.edu/) (Zuker, 2003). The software MiRNAassist was applied to improve the analysis efficiency (Xie et al., 2007).

Prediction of *A. formosa x A. pubescens* miRNAs

The prediction procedure is shown in Figure 1. The sequences of known plant miRNAs were used as query sequences for Basic Local Alignment Search Tool (BLAST) search against the EST database, with the BLASTN parameters Evalue being 1000 and word-match size between the query and database sequences being 7. Mature miRNA sequences should be no less than 16 nt and the mismatches should be less than 4. Wherever available, precursor sequences of 400 nt were extracted (200 nt upstream and 200 nt downstream to the BLAST hits) and used for the hairpin structure prediction. If the length of a sequence was less than 400 nt, the entire available sequence was used as a miRNA precursor sequence. These precursor sequences were screened by BLASTx online to reject the protein coding sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The retained precursor sequences underwent hairpin structure prediction through web server mfold. Only those meeting the following criteria were designated as miRNA homologs: (1) RNA sequence folding into an appropriate stem-loop hairpin secondary structure; (2) a mature miRNA sequence located in one arm of the hairpin structure; (3) predicted mature miRNAs with no more than 3 nt substitutions as compared with the known miRNAs; (4) miRNAs having less than 6 mismatches with the opposite miRNA* sequence in the other strand; (5) no loop or break in miRNA* sequences; (6) predicted secondary structures having higher minimal folding free energy index (MFEI)(absolute value), which usually being over 0.85 (Zhang et al., 2006b). Also, the AU content of pre-miRNA should be between 30 and 70% (Xie et al., 2007).

Prediction of miRNA targets

The near-perfect complementarity of plant miRNAs for their targets allows for very accurate prediction of miRNA targets (Fahlgren et al., 2010). MiRNA targets prediction was performed by aligning the predicted miRNA sequences with EST sequences of *A. formosa x A. pubescens* via the BLASTN program. The targets were screened according to these criteria: the number of mis-matches should be less than 4 and no gaps were allowed at the binding site. The predicted target ESTs for each miRNA family were also aligned against one another in order to eliminate redundancies (ESTs that shared greater than 98% sequence identity, usually due to separate annotations of alternative splicing products of the same locus). After removal of the repeated sequences, the function of the potential target genes were predicted by BLASTX against non-redundant protein sequences database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Identity > 25%).

Phylogenetic analysis of the new miRNAS

Considering the conservation of miRNAs and their precursors, the precursor sequences of the novel and the known miRNAs in the same family were aligned and phylogenetically analyzed by ClustalW online to investigate their evolutionary relationships (http://www.clustal.org/).

RESULTS AND DISCUSSION

Identification of *A. formosa* x *A. pubescens* miRNAs

Sequence and structure homologies are the main theory behind the computer-based approach for miRNAs prediction. As described in Materials and Methods, after BLASTN searches, all BLAST hits except coding sequences were maintained for secondary structure analysis, only those in line with the screening criteria were selected as candidates. At the end, 12 potential *A.*

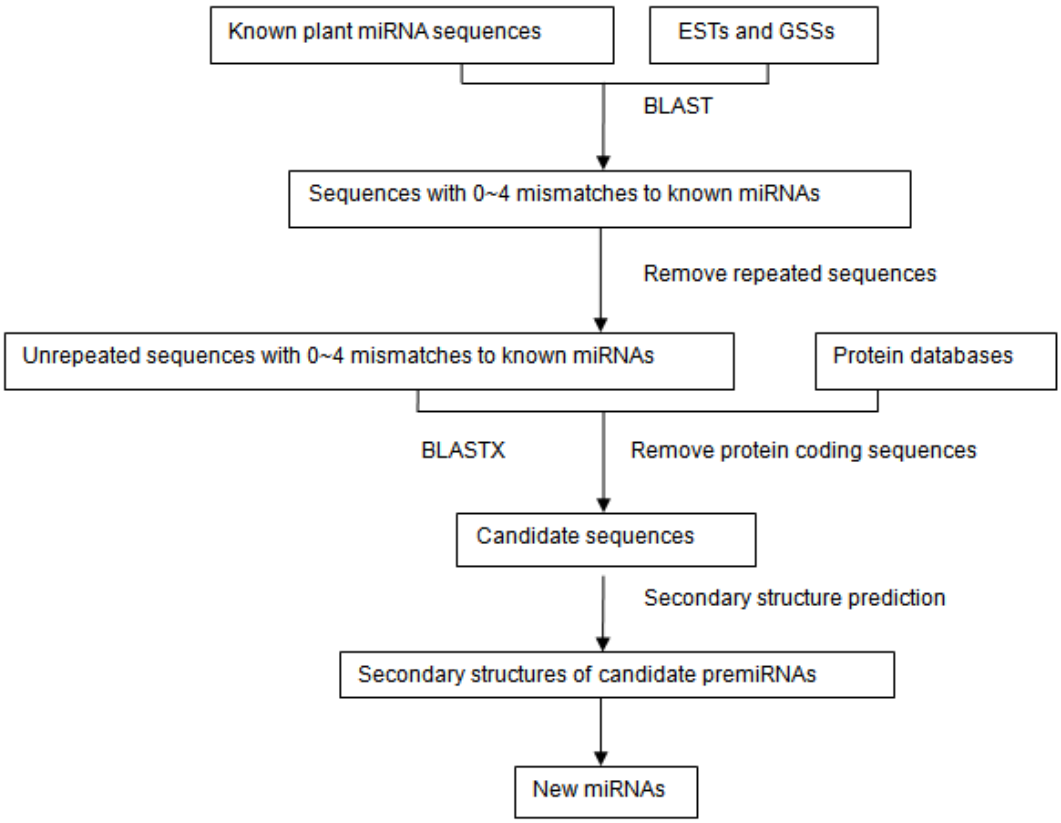


Figure 1. Flowchart of miRNA prediction.

Table 1. New miRNAs identified in *A. formosa x A. pubescens.*

New miRNA	Gene ID	MiRNA sequence	Nm/nt	Lm/nt	LP/nt	Location	A + U (%)	MFEI
aqx-miR 160a	71715431	UGCCUGGCUCCCUGUAUGCC	1	20	80	5	0.488	1.16
aqx-miIR 160b	71715431	UGCCUGGCUCCCUGUAUGCCA	1	21	80	5	0.488	1.16
aqx-miR 395	71699148	CUGAAGGGUUUGGAGGAACUC	0	21	70	3	0.571	0.99
aqx-miR414a	71689483	UCAUCUUCAUCAUCGUCAUCU	0	21	147	5	0.585	0.96
aqx-miR414b	71708459	UCAUCUUCAUCAUCGUCAUCU	0	21	140	5	0.579	0.90
aqx-miR414c	74523650	UCAUCAUCAUCAUCAUCAUCA	2	21	92	5	0.63	0.91
aqx-miR1134	74541343	CAACAACAACAACAACAACAAGAU	3	24	122	3	0.664	0.90
aqx-miR2275a	75460151	UUUGGUUUCCUCCAAUAUCUCA	0	22	104	5	0.644	0.88
aqx-miR2275b	75460151	UUUGGUUUCCUCCAAUAUCUCA	0	22	82	3	0.622	1.07
aqx-miR2275c	75460151	UUUAGUUUCCUCCAAUAUCUUA	3	22	79	3	0.633	1.01
aqx-miR2275d	71720602	UUCAAUUUCCUCUAAUAUCUCA	3	22	66	3	0.682	1.37
aqx-miR 2275e	71682697	UUCAUUUUCCUCCAAUAUCUUA	3	22	69	3	0.667	1.03

NM, Number of mismatch with the known miRNA; LM, length of mature miRNA; LP, length of precursor based on its secondary structure; MFEI, minimal folding free energy index (absolute value); Gene ID, gene from Genbank; A + U(%), the content of A and U in the precursor.

formosa x A. pubescens miRNAs belonging to 5 miRNA families were identified and named according to Meyers et al. (2008). Information on predicted miRNAs, including names, lengths, sources and other aspects, are listed in Table 1. The length of the novel miRNAs ranged from 20 to 24 nt, while the predicted precursor sequences ranged in length from 66 to 147 nt, all forming into typical stem-loop structures with the mature miRNA either on the 5' or 3'end (Figure 2). All the MFEIs (absolute value) of these hairpin structures were over 0.85, which differentiated them from other RNAs (Zhang et al., 2006b).

A. formosa x A. pubescens miRNA precursors were

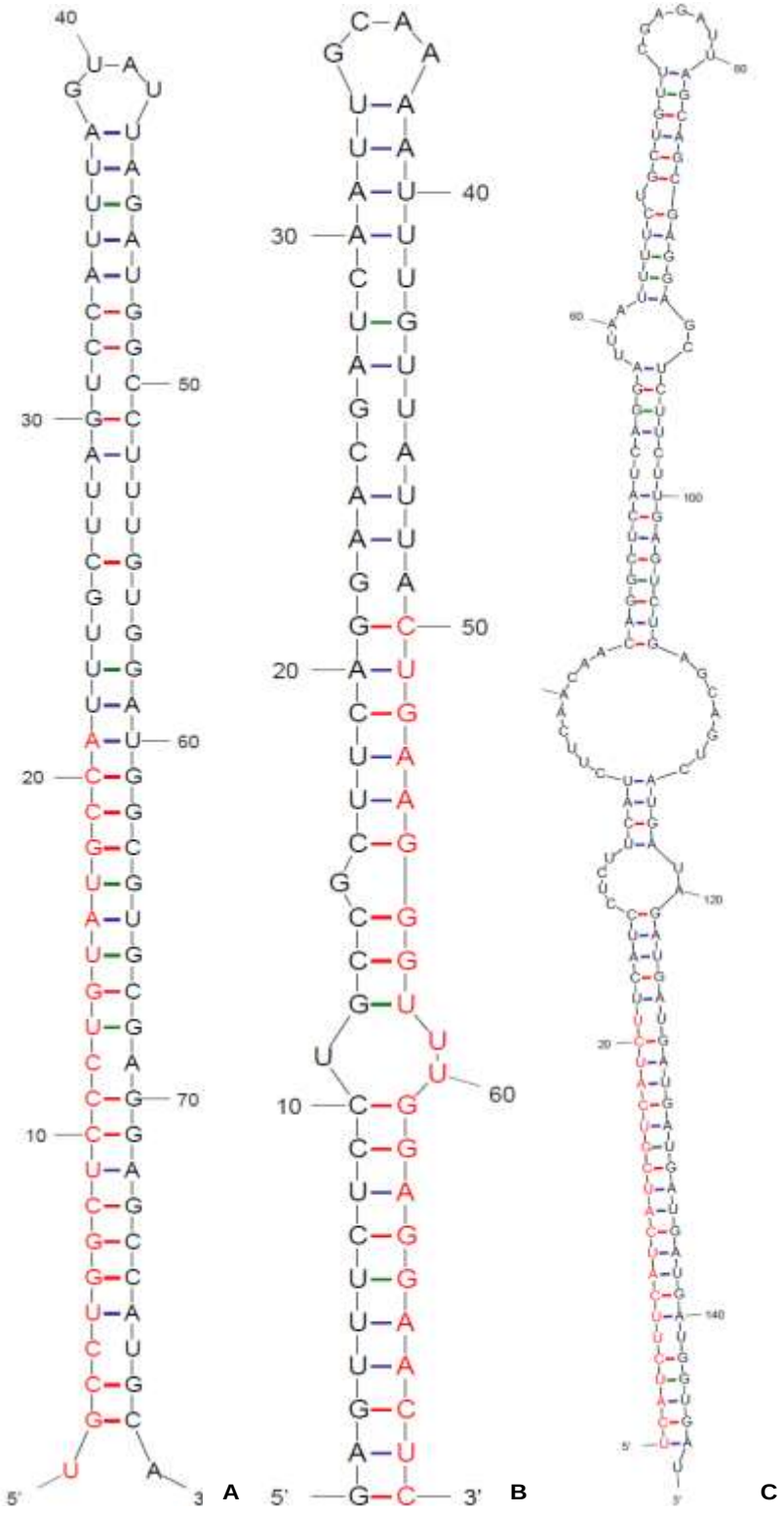
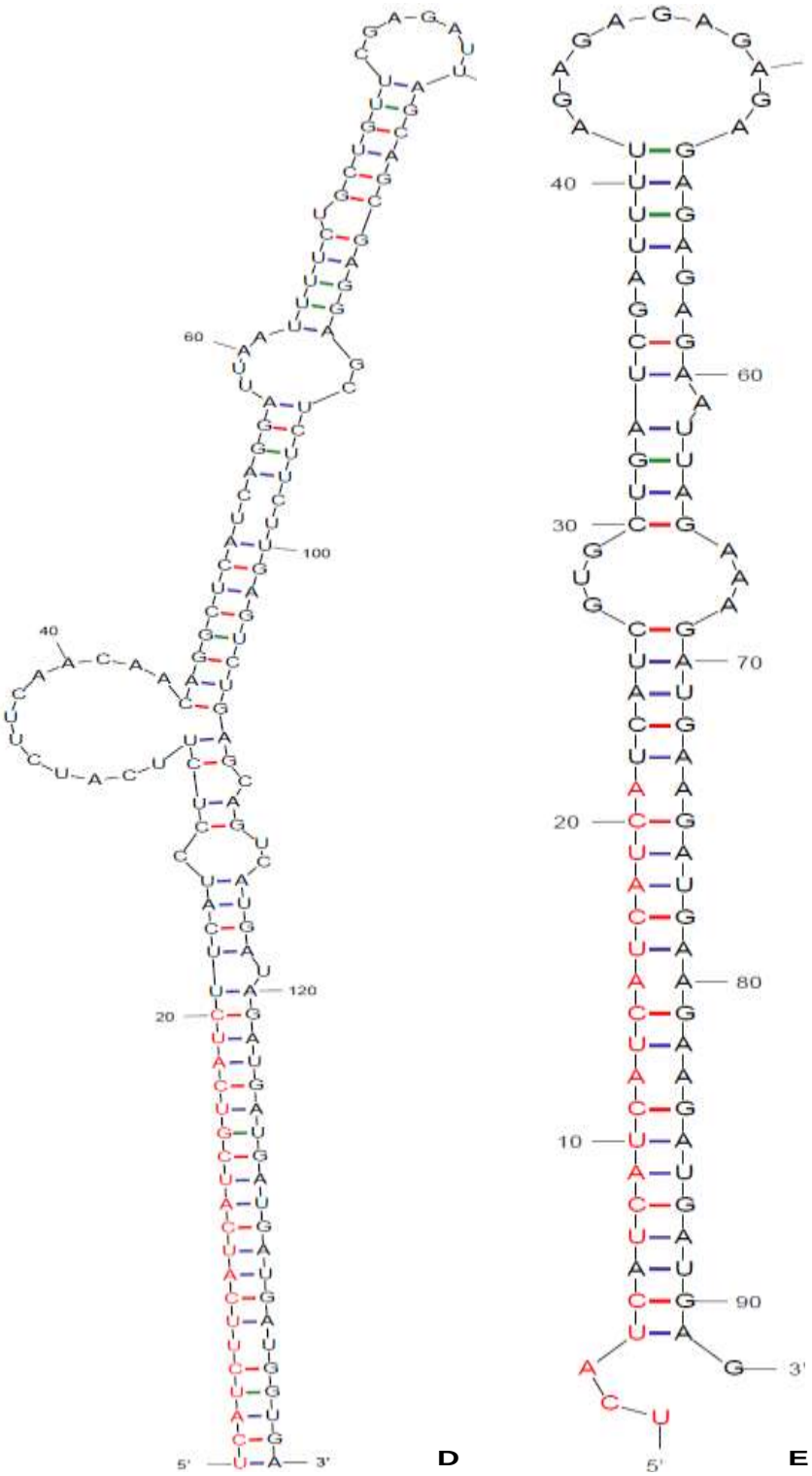


Figure 2. Secondary structures of new miRNA precursors of *A. formosa x A. pubescens* calculated by web server mfold (http://mfold.bioinfo.rpi.edu/). A, aqx-miR160a/b precursor; B, aqx-miR395 precursor; C, aqx-miR414a precursor; D, aqx-miR414b precursor; E, aqx-miR414c precursor; F, aqx-miR1134 precursor; G, aqx-miR2275a precursor; H, aqx-miR2275b precursor; I, aqx-miR2275c precursor; J, aqx-miR2275d precursor; K, aqx-miR2275e precursor. The red sequence in each precursor is the mature miRNA.



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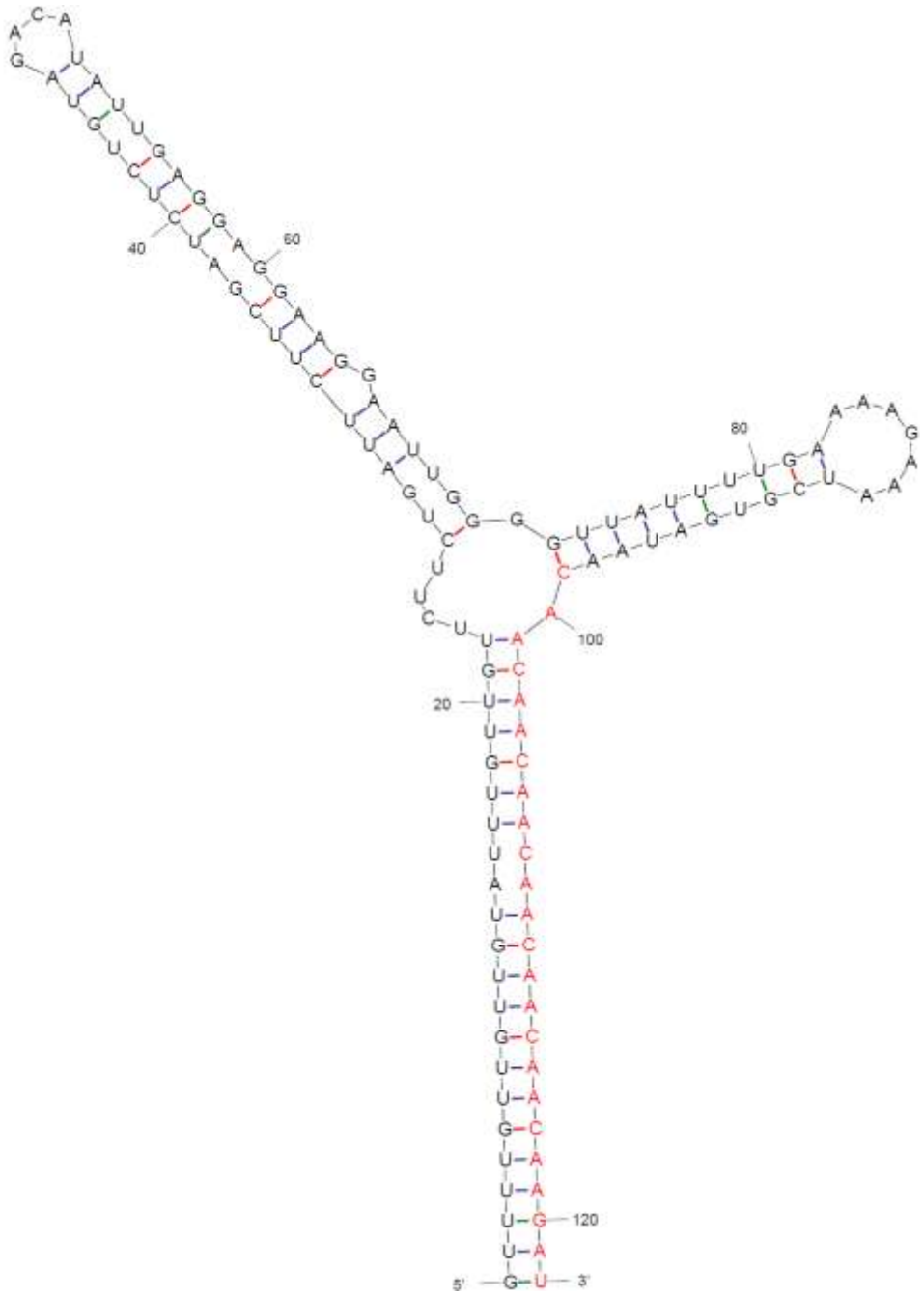
diverse in structure and size, even if they were from the same family, such as those from miR414 and miR2275 families(Figure 2), which was consistent with the diversity of miRNAs in other plants (Zhang et al., 2006a). According to Zhang et al. (2006a), about 10000 ESTs contained one miRNA. The number and sorts of miRNAs predicted in this work showed that this software-based approach was as feasible and effective as in other work



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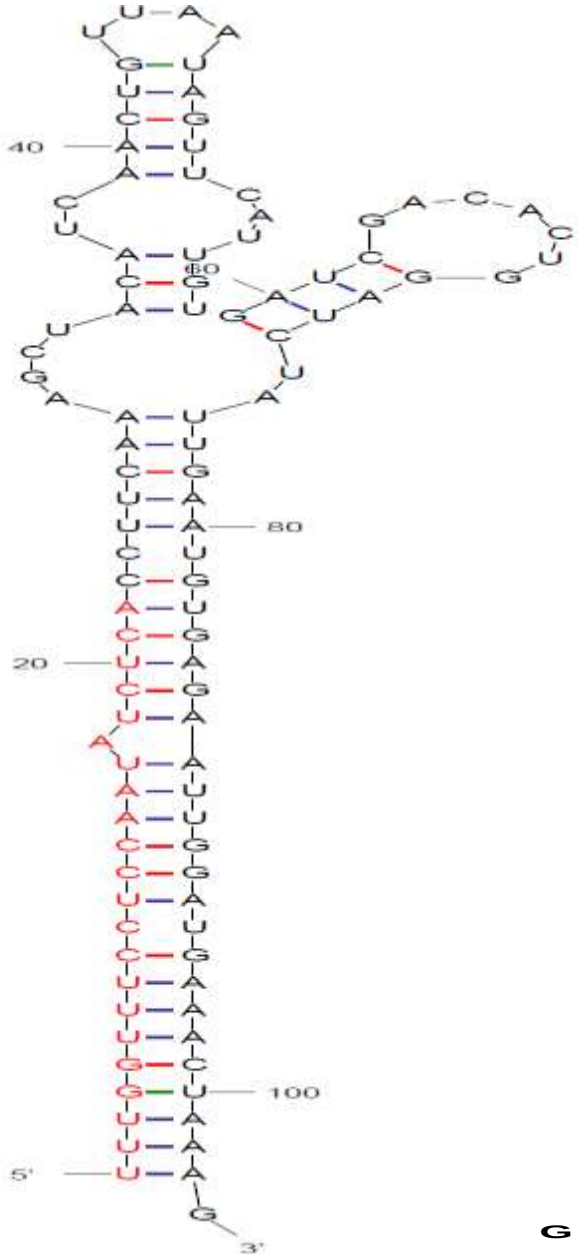
(Lu and Yang, 2010b; Han et al., 2009; Zhang et al., 2009; Song et al., 2009; He et al., 2008; Xie et al., 2007; Zhang et al., 2008).

Prediction of *A. formosa* x *A. pubescens* miRNA targets

In order to deduce the function of the novel miRNAs, their target genes were searched from the *A. formosa x A. pubescens* EST database based on the homology between miRNAs and their target mRNAs via BLASTN. A total of 51 potential targets for 12 *A. formosa x A. pubescens* miRNAs were identified and these potential miRNA targets belonged to a number of gene families that had different biological functions. The miRNAs and their putative candidate targets are listed in Table 2. The number of predicted targets per miRNA varied much,



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none were predicted for miR1134, which might be due to the low coverage of the database; as many as 43 were predicted for miR414, which indicated its vital roles in metabolic regulation. It was even reported in *Triticum aestivum* that 120 target genes were predicted for miR414 (Han et al., 2009). Many of these targets were transcription factors that might play roles in quite diverse physiological processes (Table 2).

In addition to the transcription factors, another important part of the predicted targets were various kinds of enzymes such as mannosyltransferase, sulfurylase and ubiquitin-protein ligase, which might participate in various metabolic pathways (Henquet et al., 2008; Zhou et al., 2010). Target identification is an effective way to assess and define the putative function for a miRNA in plants. EST database searches play a vital role in the discovery of miRNA targets in plants based on the homology between miRNA and its target sequences (Fahlgren and Carrington 2010).

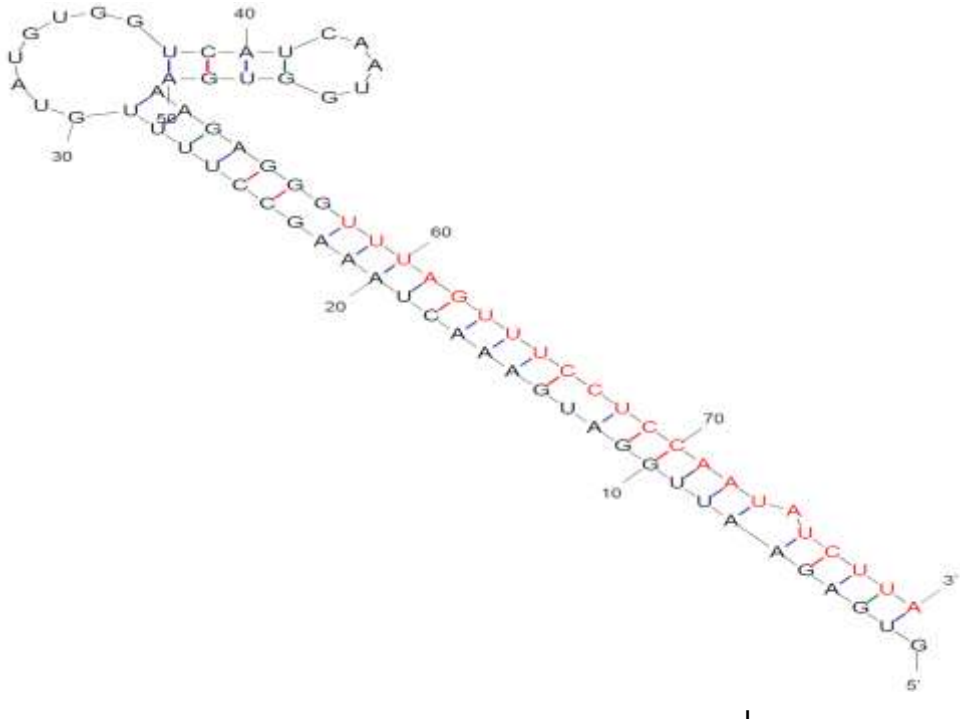
In evolutionary biology, an adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage (Gavrilets and Vose, 2005). That is, an array of species from a recent single ancestor exhibit different morphological and physiological traits to adapt to various environments. Species in the genus *Aquilegia* have spectacularly different floral morphologies with specializations to different pollinators. Also, they differ radically in their habitats ranging from shady woodlands to sun baked meadows. Considering the importance of *Aquilegia* in ecological and evolutionary studies, a much deeper understanding of the evolution of morphological, physiological and biochemical innovations at the molecular level is desirable.

MiRNAs control many important aspects of plant development, so an analysis on distribution of miRNAs in different *Aquilegia* species might give an explanation to their distinctions. In this study, besides the miRNAs found in *A. formosa x A. pubescens*, 2 miRNAs of *A. vulgaris*, one of *A. formosa* were also found from GSS database with the same method described in materials and methods (Table 3, Figure 3). *A. formosa* is one of the progenitors of the hybrid *A. formosa x A. pubescens*, but the miRNA of it was not found in the hybrid, which might be due to genomic changes after hybridization, including chromosomal rearrangements or gene silencing. Certainly, the low coverage of genomic sequences of *A. formosa x A. pubescens* might be another reason.

Sequence and expression divergence of miRNAs in closely related species and interspecific hybrids may affect miRNA accumulation and target regulation, leading to developmental changes and phenotypic variation, which has been observed in *Arabidopsis* and their interspecific hybrids (Ha et al., 2009). Though we could not draw the same conclusion now due to limited genomic resources of *Aquilegia* species, with the availability of their complete genome sequences, a detailed miRNA distribution will shed light on their morphological and ecological differences.

Phylogenetic analysis of the new miRNAs

MiRNAs are significant phylogenetic markers because of their astonishingly low rate of evolution (Liu et al., 2010). Besides, they are being looked upon as a possible solution to outstanding phylogenetic problems. Considering the special phylogenetic position of *Aquilegia*, it is desirable to make an analysis with miRNAs in a phylogenetic context. The distribution of miRNAs in *Aquilegia* and some other models such as *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and *Physcomitrella*



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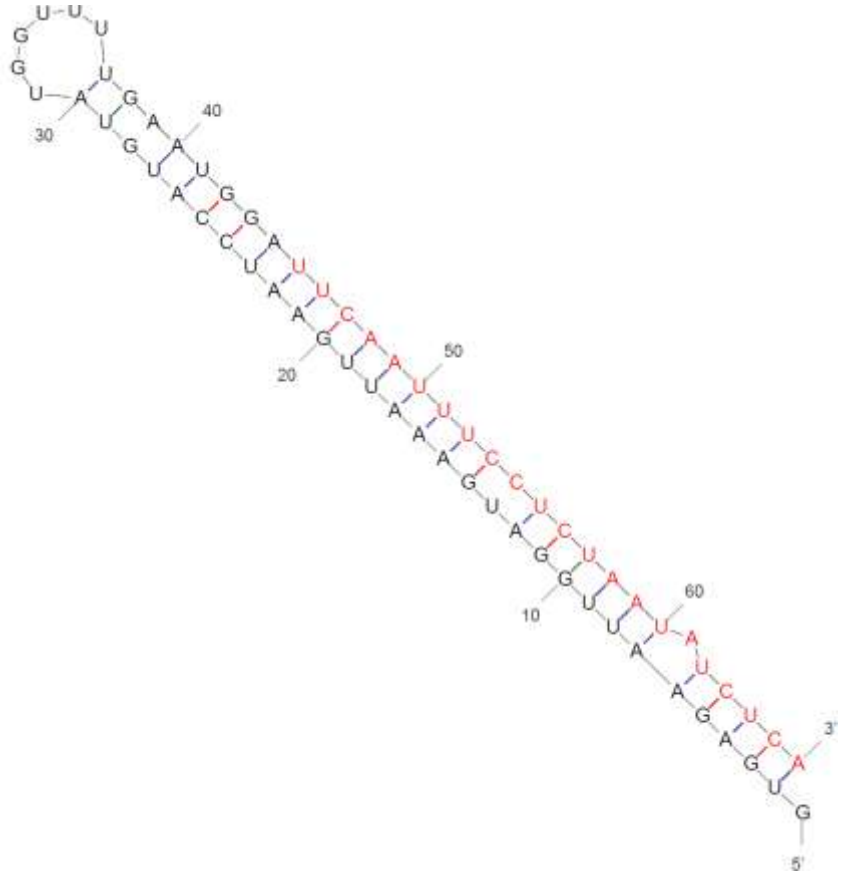

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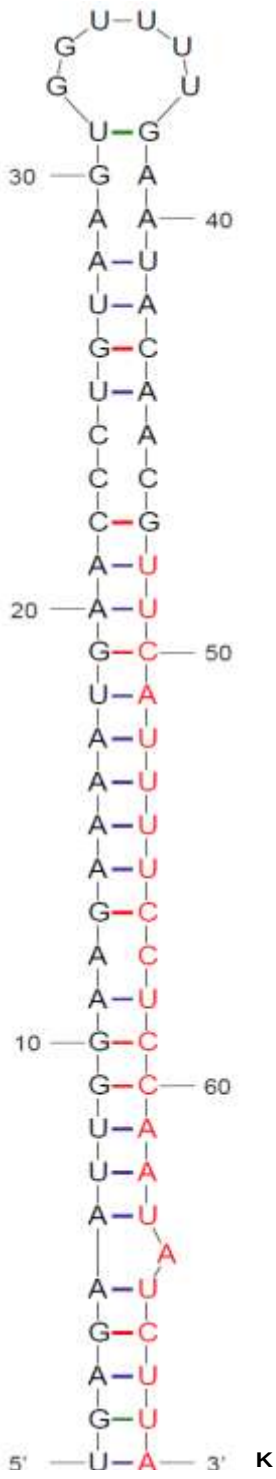

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patens indicated that, a small group of miRNA families such as miR160, miR164, miR395 and miR399 appear to be highly conserved across these species (Table 4).

The conservation of mature miRNAs and their precursors provides the chance to investigate their evolutionary relationships. Nearly the same conclusion could be drawn from the phylogenetic trees that, *Aquilegia* occupied an intermediate phylogenetic position between core eudicot (*Arabidopsis*) and monocot (*Oryza*) model species, with more close relationship to *Arabidopsis* (Figure 4), that is highly consistent with the plant taxonomy (Puzey and Kramer, 2009). Also it could be seen that members in the same family of a species were usually distantly related, which suggested that different miRNA genes might evolve at different rates, but it was not always the case of *Aquilegia*, for example, members in miR395 and miR399 families were all closely related, indicating sharing common ancestor, that provided cogent molecular evidences for adaptive radiation of this genus (Figure 4).

Though the mature miRNA sequences are almost invariable, the sequences outside the mature miRNAs are highly variable, which suggest an important role of secondary precursor structure in miRNA processing and biogenesis, allowing generation of the same miRNAs to take on novel spatial and temporal functions (Ha et al., 2008). Earlier studies showed that, miR2275 was only found in monocots such as *Z. mays* and *O. sativa*. This was the first time to report it in eudicots. In *A. formosa x A. pubescens,* 3 members of miR2275 family originated from the same transcript, 2 of which share the same mature miRNA sequences but generated from different precursors (Figure 5). Unlike animal miRNA gene clusters, plant miRNA genes of the same family are often scattered throughout the genome, although clustering seems uncommon in some plants such as soybean (Zhang et al., 2008). The consequence of the co-transcription of similar or identical miRNA genes on a plant gene cluster would be a dosage effect (Li and Mao, 2007).

Conclusions

In this work, 12 miRNAs were identified from the EST database of *A. formosa x A. pubescens* and 51 potential targets of them were predicted, which were related to different physiological processes. Besides, 3 miRNAs were predicted from the GSS databases of *A. formosa* and *A. vulgaris*. A phylogenetic analysis provided evidences for the phylogenetic position of *Aquilegia*, as well as its adaptive radiation at molecular level. Above all, the findings from this study will contribute to further researches of miRNA functions and regulatory mechanisms in *A. formosa x A. pubescens* and will also help in the understanding the genetic basis of evolutionary and ecological characteristics of *Aquilegia*.

ACKNOWLEDGEMENTS

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Table 2.The potential targets of novel miRNAs in *A. formosa x A. pubescen.*

miRNA	Targeted gene (ID)	Targeted protein	Possible function
miR-395	75452677	ATP sulfurylase	metabolism
	71699148	hypothetical protein	unknown
miR-414	75453910	negative cofactor 2 transcriptional co-repressor	transcription
	75459757	hypothetical protein	unknown
	74560612	nuclease, putative	DNA degration
	74564613	alpha DNA polymerase, putative	DNA synthesis
	74549977	transcription factor	transcription
	75457050	extracellular ligand-gated ion channel	Metabolism
	75459231	hypothetical protein	unknown
	74555834	pre-mRNA-splicing factor	transcription
	74552721	hypothetical protein	unknown
	74548076	hypothetical protein acetolactate synthase large subunit	metabolism
	74552769	rac gtpase	signal transduction
	74554333	aminoacyl-tRNA synthetase	translation
	74556618	transmembrane protein2; receptor	signal transduction
	74556622	zinc finger protein	transcription
	74539548	ubiquitin specific protease 39 and snrnp assembly factor	metabolism
	74528215	hypothetical protein	unknown
	74539175	hypothetical protein	unknown
	74528345	zinc finger family protein	transcription
	74529163	transcription factor	transcription
	74533837	ubiquitin-protein ligase	metabolism
	74535247	hypothetical protein	unknown
	74539939	transcription regulator	transcription
	74531934	hypothetical protein	unknown
	74533752	ubiquitin-protein ligase	metabolism
	71722564	zinc finger protein	transcription
	74523550	transcription factor	transcription
	74513475	transcription factor	transcription
	74515034	hypothetical protein	unknown
	74519639	ABC transporter family protein	metabolism
	71713603	hypothetical protein	unknown
	71718234	translation initiation factor	translation
	71708459	alpha chain of nascent polypeptide associated complex	transcription
	71683794	f-box family protein	transcription
	71681830	ribosomal protein S6	translation
	71702073	SPla/RYanodine receptor domain-containing protein	metabolism
	71702182	hypothetical protein	unknown
	71703092	cell division protein	cell division
	71691704	hypothetical protein	unknown
	71695172	cholinephosphate; cytidylyltransferase	metabolism
	75455214	CONSTANS-like zinc finger protein	transcription
	74552739	DNA binding protein; chromatin remodeling factor subunit	transcription
	74539548	ubiquitin specific protease 39 and snrnp assembly factor, putative	metabolism
	74529163	transcription factor	transcription
	74539939	transcription regulator	transcription
	71722564	zinc finger protein	transcription
	74523550	transcription factor	transcription
	71716392	mannosyltransferase; glycosyltransferase	metabolism
	71703679	ribosomal protein S6	protein synthesis

Table 2. Cont..

miR-2275	71687376	esterase/lipase/thioesterase family protein; hydrolase, alpha/beta fold family protein	metabolism

One ID number was listed when a targeted gene had multiple IDs due to separate annotations of alternative splicing products: ID, indicate the gene in Genbank.

Table 3. New miRNAs identified in *A. formosa* and *A. vulgaris.*

New miRNA	Gene ID	miRNA sequence (5′3′)	NM/nt	LM/nt	LP/nt	Location	A + U (%)	MFEI
aqv-miR164a	191174909	UGGAGAAGCAGGGCACGUGCA	0	21	67	5	0.552	1.22
aqv-miR164b	191174908	UGGAGAAGCAGGGCACGUGCC	1	21	67	5	0.478	1.04
aqf-miR399	254537223	UGCCAAAGGAGAGUUGCCCUA	0	21	80	3	0.545	1.02

NM, Number of mismatch with the known miRNA; LM, length of mature miRNA; LP, length of precursor based on its secondary structure; MFEI, minimal folding free energy index(absolute value); Gene ID, gene from Genbank; A + U(%), the content of A and U in the precursor.

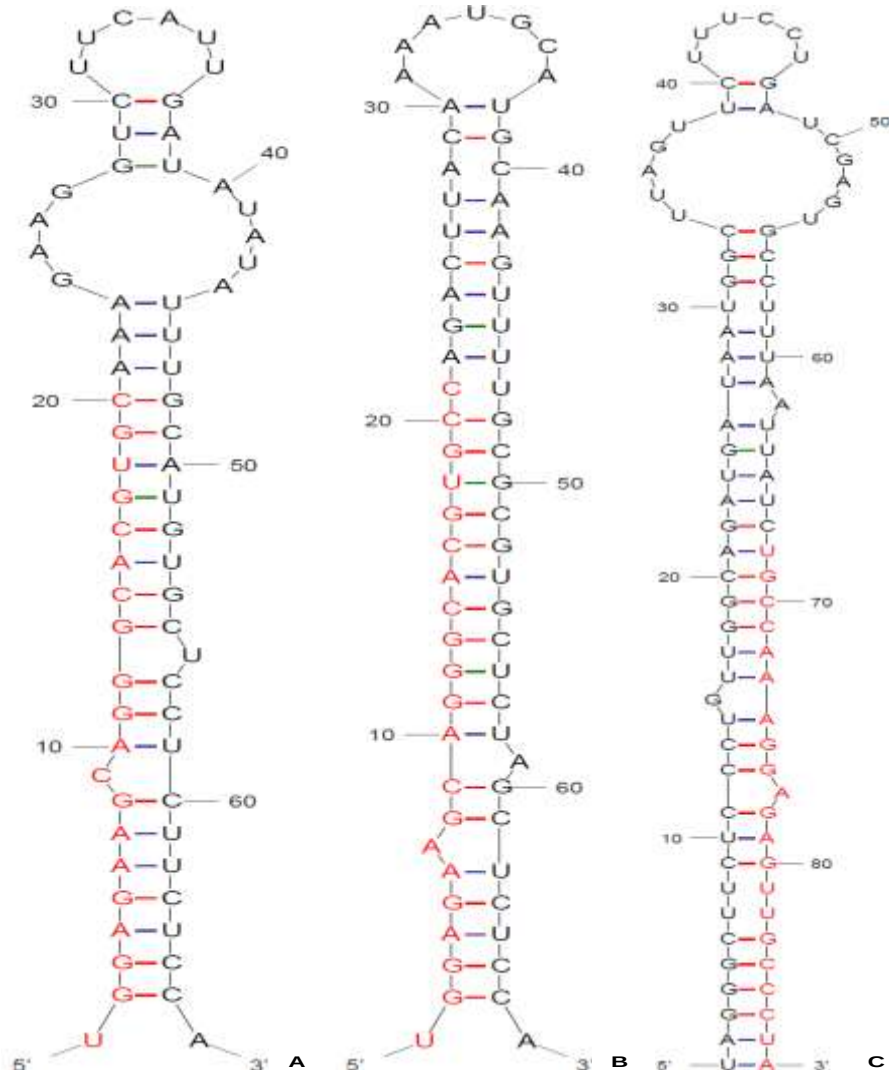


Figure 3. Secondary structures of new miRNA precursors of *A. formosa* and *A. vulgaris* calculated by web server mfold (http://mfold.bioinfo.rpi.edu/). A, aqv-miR164a precursor; B, aqv-miR164b precursor; C, aqf-miR399 precursor. The red sequence in each precursor is the mature miRNA

Table 4. Distribution of miRNAs in some plant species.

Plant species	156	159	160	162	164	166	167	168	169	171	172	319	390	393	394	395	396	397	398	399	408	414	1068	1134	1521	2275
A. thaliana	8	3	3	2	3	7	4	2	14	3	5	3	2	2	2	6	2	2	3	6	1	1				
O. sativa	13	6	6	2	6	14	10	2	16	9	4	2	1	2	1	23	9	2	2	11	1	1				2
P. patens	3		9			13	1			4		5	4			1					1	1	1			
T. aestivum		2	1		1		1			1										1	1			1		
Z. mays	9	11	8	1	8	14	10	2	17	14	5	4	2	3	2	16	8	2	2	10	2					4
A. formosa x A. pubescens			2													1						3		1		5
A.. coerulea	2	1	2			5	1	1	3	6	2	1			1	2	2		2	1	1					
A. vulgaris					2																					
A. formosa																				1						

Numbers in boxes represent the number of miRNAs present in a particular miRNA family.

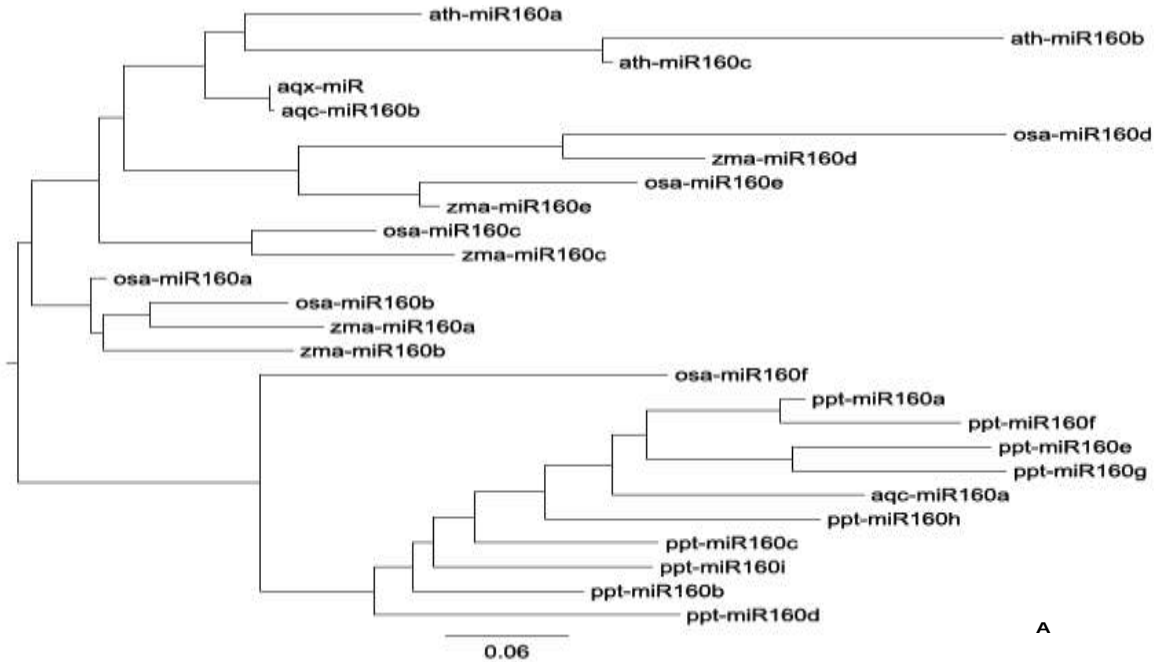
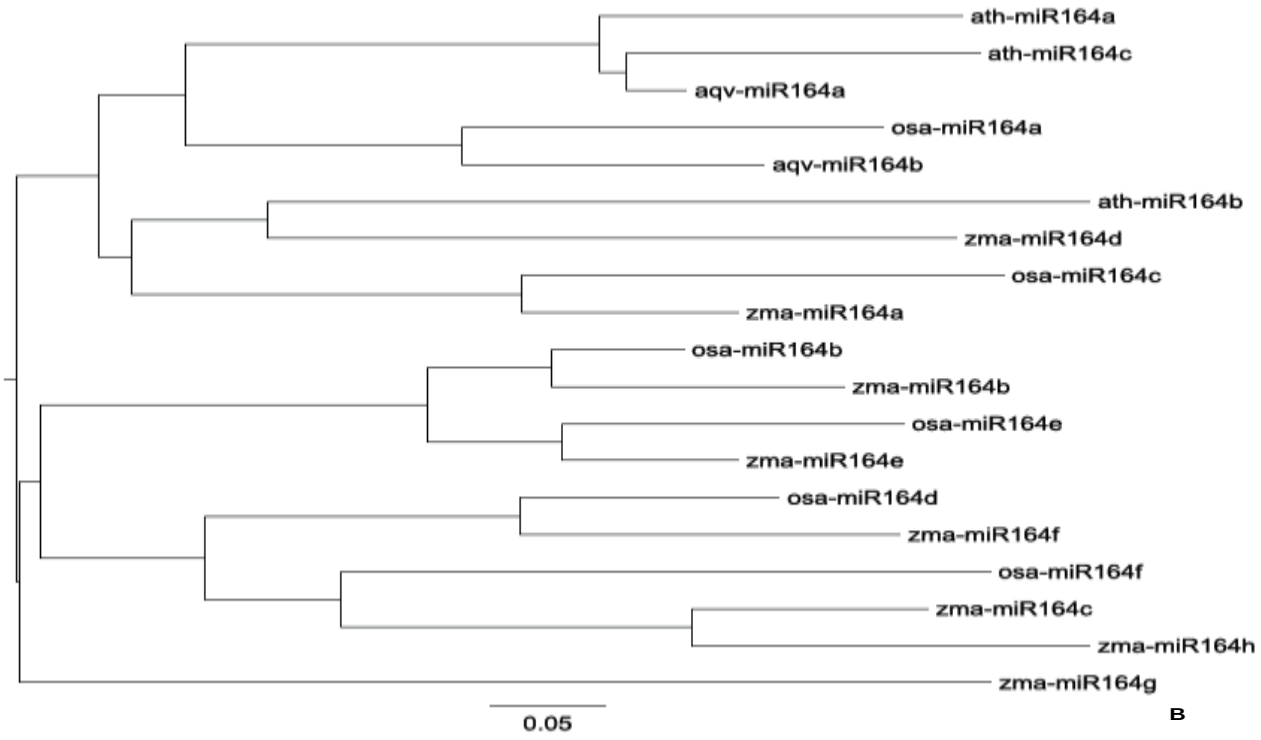


Figure 4. Phylogenetic analyses of different miRNA families. A, miR160 family; B, miR164 family; C, miR395 family; D, miR399 family.



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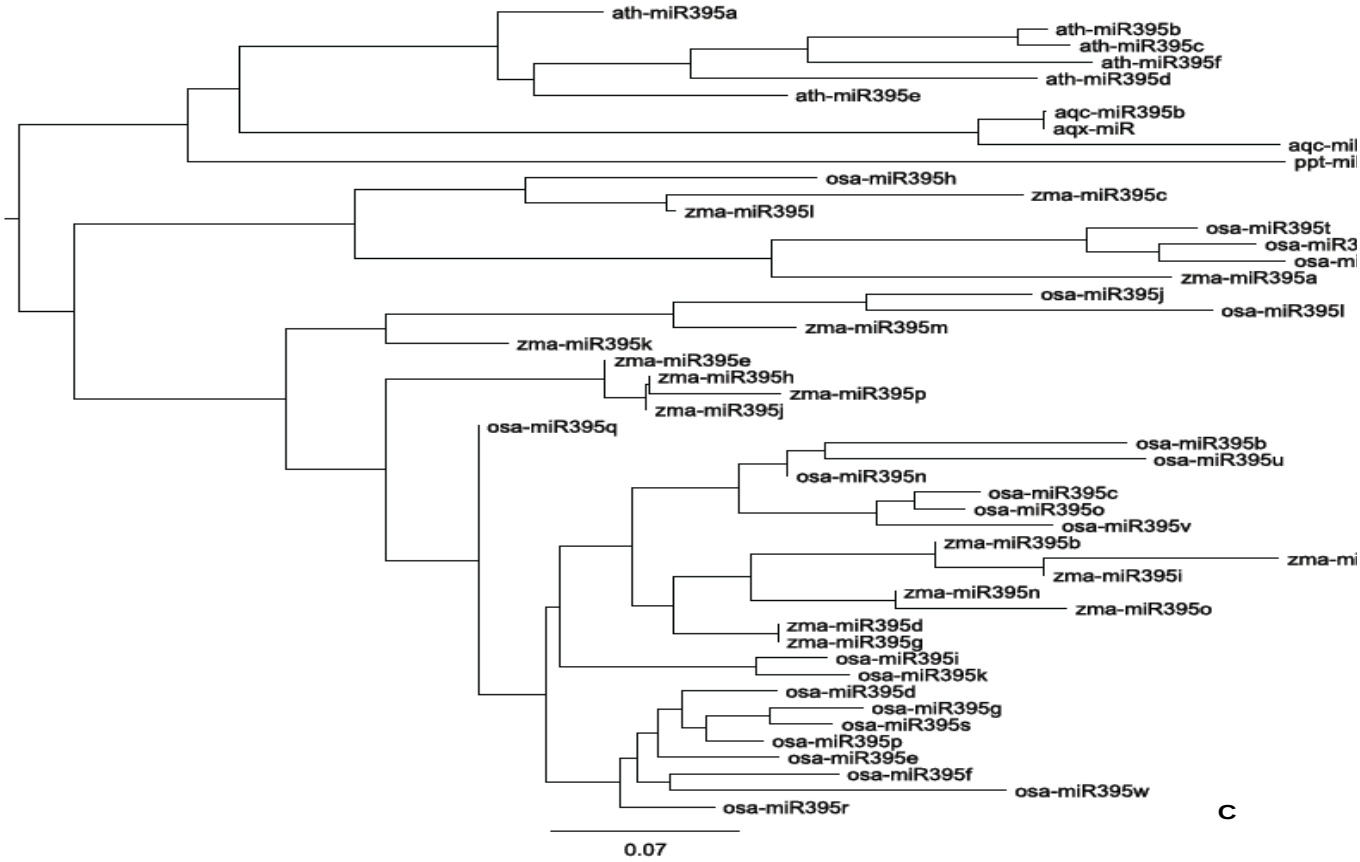

Figure 4. Contd.

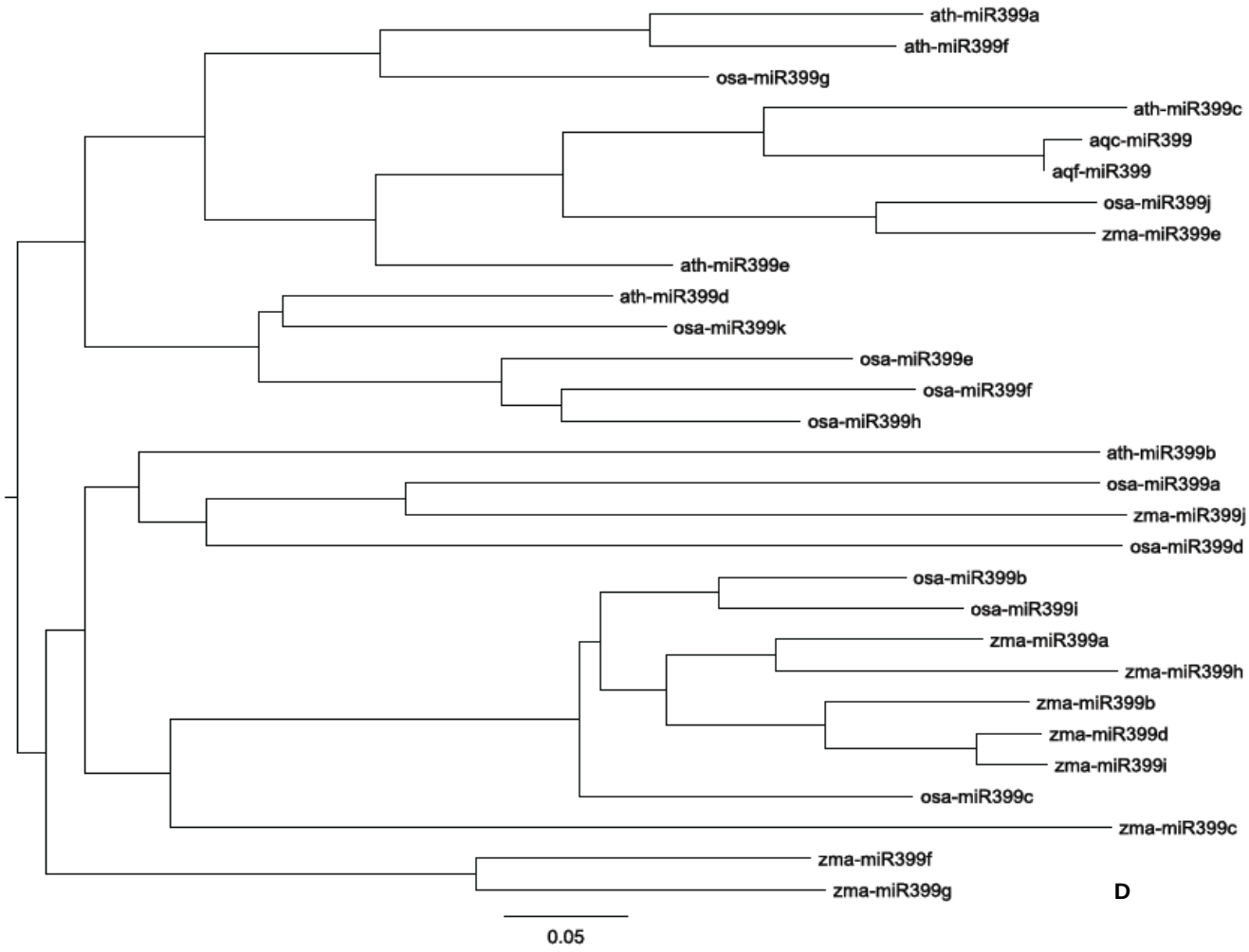

Figure 4. Contd.

5'gttttgtttaggtagcaaagttggtgtttgaatgtgaggattggatgaaaccaaactccactctagctatcttttagttgcaaaaagtaagag**tttggtttcctccaatatc**
tcaccttcaaagctacatcaactgttaatagttcattgtgatcgacactggatctattgaatgtgagaattggatgaaactaaagccttttgtatgtggtcatcaat
ggtgaaagagggtttagtttcctccaatatctta**c**cttcaataatctcaacttaaattctaggaggtataaattaaaaaacaattcaaatgaaatcattgtcctacattaatct
agtgatcttgatatataacagaaaaaatgtactagaacctcatatataacagaaagagatcgacaag3'

Figure 5. Location of 3 miR2275 precursors in the transcript. The blackened indicated miR2275a precursor; the underlined indicated miR2275b precursor; the double-underlined indicated miR2275c precursor.

MiRNAassist software.

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