

Original Research Paper

Mining of miRNAs using Next Generation Sequencing (NGS) data generated for Okra (*Abelmoschus esculentus*)

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ABSTRACT

MicroRNAs (miRNAs) are small, highly conserved non-coding RNA molecules involved in the regulation of gene expression in eukaryotes. Gene expression involves post-transcriptional gene regulation by miRNAs. miRNAs are formed from precursor RNA molecules that fold into a stem loop secondary structure. The mature miRNA is one end of the precursor miRNA, defined by the cut from ‘Drosha’ on either the 5’ or 3’ arm. In this study, we have used a bioinformatics approach to identify miRNAs in 3,361 contigs obtained from partial genome sequence data of *Abelmoschus esculentus* (okra) sequenced by NGS technology. Using C-mi and psRNA Target tools, we identified two miRNAs and their target RNAs for which a regulatory miRNA binding has been verified. Their targets consisted of transcription factors involved in growth and development, gene regulation and metabolism. Phylogenetic analysis of the newly identified miRNA family has been done to compare their level of conservation with respect to the other members of the plant kingdom.

Keywords: microRNA; Next Generation Sequencing; *Abelmoschus esculentus*; miRNA targets.

INTRODUCTION

The micro RNAs (miRNAs) were discovered by Victor Ambros in *C. elegans* 1993 (Lee *et al.*, 1993). MicroRNA (miRNAs) belongs to a class of single stranded RNA molecules, ranging between 18-22 nucleotides length, containing a non-coding sequence. They are known to have an important role in post-transcriptional regulation (Jia *et al.*, 2010). The RNA interferences are mediated by miRNAs as well as siRNAs by binding to the 3’-UTR of target mRNAs which modulates the cell functions in all tissues following the development and differentiation (Lee *et al.*, 2004; Bartel 2004; Ying and Lin, 2005). The length of a primary miRNA gene transcript is about 1Kb long with a 5’-cap and poly-A-tail (Lee *et al.*, 2004). A microprocessor complex comprising a class II endoribonuclease III Drosha cuts the pri-miRNA into a shorter (60-70bp) precursor miRNA (pre-miRNA) (Lee *et al.*, 2002). After the precursor is actively transported into the cytoplasm, another endoribonuclease and Dicer cuts off the loop region and produces a ~22bp long double stranded RNA, which contains the mature miRNA and its complementary strand called miRNA* (Lau *et al.*,

2001). Mature miRNAs are single-stranded RNA molecules of approximately 21 nucleotides (nt) in length processed from a precursor molecule (pre-miRNA).

miRNA genes of the known eukaryotic genomes are about 1-2%, which constitutes an important class of regulators involved in several developmental, physiological and disease-associated cellular processes (Lu *et al.*, 2010). In plants, miRNAs target mostly the transcription factors and are implicated in plant growth and development (Perez-Quintero *et al.*, 2010). Most miRNA genes in eukaryotes are well characterized and are found in the intergenic regions or in anti-sense orientation of certain genes containing their own miRNA gene promoter and regulatory units (Nabajit., 2012). MicroRNAs are named using the “miR” prefix and a unique identifying number (e.g., miR-1, miR-2, . . . miR-89) and the genes that encode the miRNA are also named using the same three-letter prefix, with capitalization, hyphenation, and italics according to the conventions of the organism (Ambros *et al.*, 2003).

The growing interest in miRNAs has attracted the attention of many scientists to identify hundreds of

plant miRNAs and their targets based on experimental and computational approaches and the majority of studies were focused mainly on the model plants: *Arabidopsis thaliana* and rice (Griffiths-Jones 2004; Griffiths-Jones *et al.*, 2006). It is known that the mature miRNAs are about 21-23 nucleotides long and have been found in the wide range of eukaryotes, from *Arabidopsis thaliana* and *Caenorhabditis elegans* to mouse and human (Bartel., 2004).

However, the genes coding miRNAs are much longer than mature miRNAs; they usually contain several 10s of nucleotides to 100s of nucleotides and the length of miRNA genes varies from miRNA to miRNA and even from species to species. For example, miRNA genes in plant species are usually longer than in animals (Zhang *et al.*, 2007). Computational methods have been an invaluable tool that can complement experimental approaches to understand the biology of miRNAs. Although there are several miRNA and target prediction tools available, (Bartel, 2009; Mendes *et al.*, 2009), the number of tools customized for plant miRNA and target analysis are few in number (Numnark *et al.*, 2012). Further, a majority of computational approaches are based on the whole genome sequence which makes it a difficult task to identify the miRNAs in a wide range of species (Zhang

et al., 2006c; Brown and Sanseau, 2005). In our study the new miRNAs and their targets identified in okra using some bioinformatics tools could improve our understanding of the possible roles of miRNAs in regulating the growth and development of *A. esculentus* (okra).

Abelmoschus esculentus (okra) belonging to the Malvaceae family is one of the polysaccharide rich plants, which is known by many local names in different parts of the world viz. lady's fingers, bhindi, gumbo etc. This is one of the most important vegetables, which is widely grown well in both tropical and temperate zones. This plant is popular for its mucilaginous properties and acclaimed to have various health benefits which include anti-diabetic properties (Amin, 2011).

In this study, the comparative genomics of NGS sequences of *A. esculentus* and known plant miRNAs have been used (i) to predict microRNA genes in okra (ii) to identify the phylogenetic relationship of newly identified miRNAs with existing plant miRNAs and (iii) to identify the secondary structure, folding of precursor microRNAs, and its target genes.

MATERIALS AND METHODS

The methodology followed in this study is briefly presented in the flow chart (**Figure 1**)

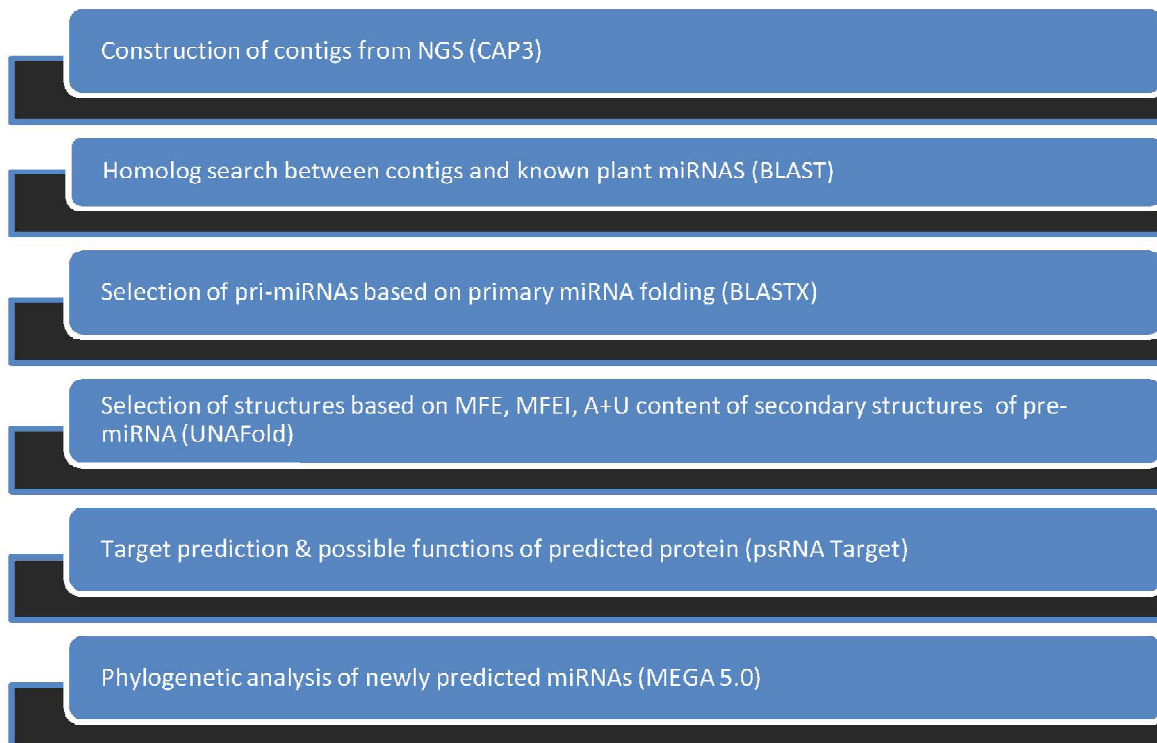


Fig 1. Methodology for miRNA and target prediction

Source of *A.esculentus* sequences

miRNAs of *Abelmoschus esculentus* and their target genes were identified by analyzing NGS data. Genomic DNA sequences of *A. esculentus* in fasta format were obtained from the NGS sequencing of total genomic DNA using the Roche 454 platform. Contigs were developed using CAP3 software (Huang and Madan, 1999) and these contigs were used for further analysis.

Prediction of miRNA in *Abelmoschus esculentus*

We have used miRNA identification tool C-mii (<http://www.biotech.or.th/isl/c-mii>) which can predict plant miRNAs and targets in plants (Numnark *et al.*, 2012). It is a stand alone software package, with a graphical user interface, which can be used for identifying and analyzing plant miRNAs and targets. The required software for the package viz. BLAST, Java Development Kit (JDK), Perl, Python, UNAFold, and Ghost script are included in the package which are installed during the installation of C-mii. C-mii identifies plant miRNAs in a step-by-step filtering procedure; the user can examine the results after each step and customize the parameters for the next step. miRNA identification pipeline consists of the successive implementation of the following steps: sequence loading and authentication, homolog search, primary miRNA folding and precursor miRNA folding. The three filtering steps to determine the miRNA are described as below.

Homolog Search

Upon the identification of an ever increasing number of plant miRNA genes in several species, homology-based search methods began to be developed seeking the complete enumeration of miRNAs in model organisms (Li *et al.*, 2005; Dezulian *et al.*, 2006). Homolog genes are genes related to each other by descent from a common ancestral DNA sequence (Kiezun *et al.*, 2012). In this module we have loaded the 3,361 contigs data with the parameters: E-value = 0.01 and no. of mismatches (between miRNAs and miRNA*) ≤ 2 . Input sequences were scanned with BLAST to search for homologs in the plant miRNAs present in miRBase, which was pre-installed in C-mii.

Primary miRNA folding

The primary miRNA folding module predicts the secondary structure folding of those input sequences

which contain homologous miRNAs. In this module protein-coding sequences and other types of non-coding RNAs from input sequences are removed. The removal of unwanted sequence types is based on BLASTX and BLASTN searches against protein and non-coding RNA databases. The secondary structure and folding of primary miRNAs (pri miRNAs) are predicted for the remaining sequences and minimal free energies (MFEs), minimal folding free energy indices (MFEI), sequence lengths, nucleotides and GC content are calculated. After these steps the selected sequences are passed to the precursor miRNA folding module.

Precursor miRNA folding

This is the last module of the C-mii tool for miRNA prediction. The last step of the precursor miRNA folding module aids in extracting the stem-loop structures from the secondary structures of pri-miRNAs. Structures with multi-loops or mature miRNA sequences not located on one arm, will be promptly removed by C-mii. And finally the result consisting of MFEIs, MFE value, the number of mismatches between miRNA and existing miRNAs, the number of bulges, and bulge sizes etc was exported. The percentage of A+U content in precursor sequence was manually calculated. UNAFold is then reapplied to the remaining sequences to predict the secondary structure folding of precursor miRNAs (pre-miRNAs).

miRNA prediction from secondary structure

The hairpin secondary structures of two probable miRNA in *A.esculentus* obtained from C-mii were evaluated as miRNA homologs.

Some major criteria used for miRNA prediction from secondary structure are

- A mature miRNA sequence should be located in one arm of the hairpin structure
- There should be no more than 2 substitutions between mature miRNA sequence and its opposite miRNAs (Mandhan *et al.*, 2012)
- Predicted secondary structures with higher minimal folding free energy (MFE) should be e^{-18} kcal mole⁻¹ free-energy
- A+U content should be in the range of 30-70%

Computational prediction of miRNA Targets

miRNA target prediction in the plant kingdom is easier due to high and significant complementarities of miRNA - miRNA targets (Lee and Ambros, 2001). So we have identified conserved and novel miRNAs using the target prediction web server psRNA Target (Dai and Zhao, 2011). These potential miRNA targets belonged to a several number of gene families that had different biological functions. Many of these targets are transcription factors that control plant development and phase change from vegetative growth to reproductive growth (Lu and Yang, 2010).

Phylogenetic Analysis of the new miRNAs

Phylogenetic analysis is very essential in searching the evolutionary tree of any organism. Considering the large number of members present in miR399 family, we constructed a phylogenetic tree with the maximum parsimony method of miR399 family through MEGA5.0 (Tamura *et al.*, 2011). This method describes a particular non-parametric statistical method for constructing phylogenies and investigate their evolutionary relationships.

RESULTS AND DISCUSSION

Computer-based miRNA identification is becoming more advantageous than the experimental approaches because of the high efficiency and low cost. At present, the mainly used plant sequences for miRNA prediction are of three kinds through genome, GSS and EST. More than one thousand plant miRNA genes are annotated and some of them are well characterized till date (Griffiths-Jones *et al.*, 2008). There is no any functional or sequence information available on miRNA in okra which is an economically important crop in the tropics and subtropics (<http://www.vegetableipmasia.org/Crops/Okra.html>).

In our present study of miRNAs, sequence and structure properties have been identified in *A. esculentus* employing the computational approaches via C-mii software. Two miRNAs were predicted from NGS contigs whose length ranged from 19 to 21nt (Table 1). To understand the function of the identified miRNAs, their putative targets were predicted using

bioinformatics approach. Five targets for the newly identified putative miRNAs were predicted.

Newly predicted miRNAs

Homology search

Homology-based search methods are generally used for the identification of plant miRNA genes. Homolog genes are the genes which are related to each other by descent from a common ancestral DNA sequence (Kiezum *et al.*, 2012). In this module we have loaded 3,361 contigs data with the parameters: E-value = 0.01 and number of mismatches between miRNAs and miRNA* ≤ 2 . A total number of 87 sequences were found to have binding sites of mature miRNAs. Among these 87 sequences, only 29 are distinct sequences, belonging to 15 families and 30 members. All these sequences were used as input for the primary miRNA folding module.

Primary miRNA folding

As the primary miRNAs are expressed only in the embryonic stem cells, the corresponding pre and mature miRNAs will be expressed at a later developmental stage and transcript to different processing proteins (Chakraborty *et al.*, 2012). In this module we have identified the 85 sequences which have recorded in miRBase. But out of 85 sequences, 27 sequences are unique which are belonging to 15 families and 30 members. Later all these sequences were selected with Auto mode for the precursor miRNA folding module.

Precursor miRNA folding and secondary structure prediction

From the final step of the module after filtering, only 57 which have already recorded and the 3 sequences which were distinct from that sequence are belonged to 14 families and 29 members respectively. These were finalized as potential miRNAs from 3,361 contig sequences. We have considered only the criteria as like MFE, MFEI, A+T content, homolog mismatches. Following these criteria we obtained two miRNAs in okra and also predicted the stem-loop secondary structures of identified miRNAs, as well as the precursor sequences and the mature sequences by using UNA fold which is already pre-installed in c-mii. All the sequences formed into typical stem-loop structures, with the mature miRNA either on the 5' end or the 3' end (**Figure 2**).

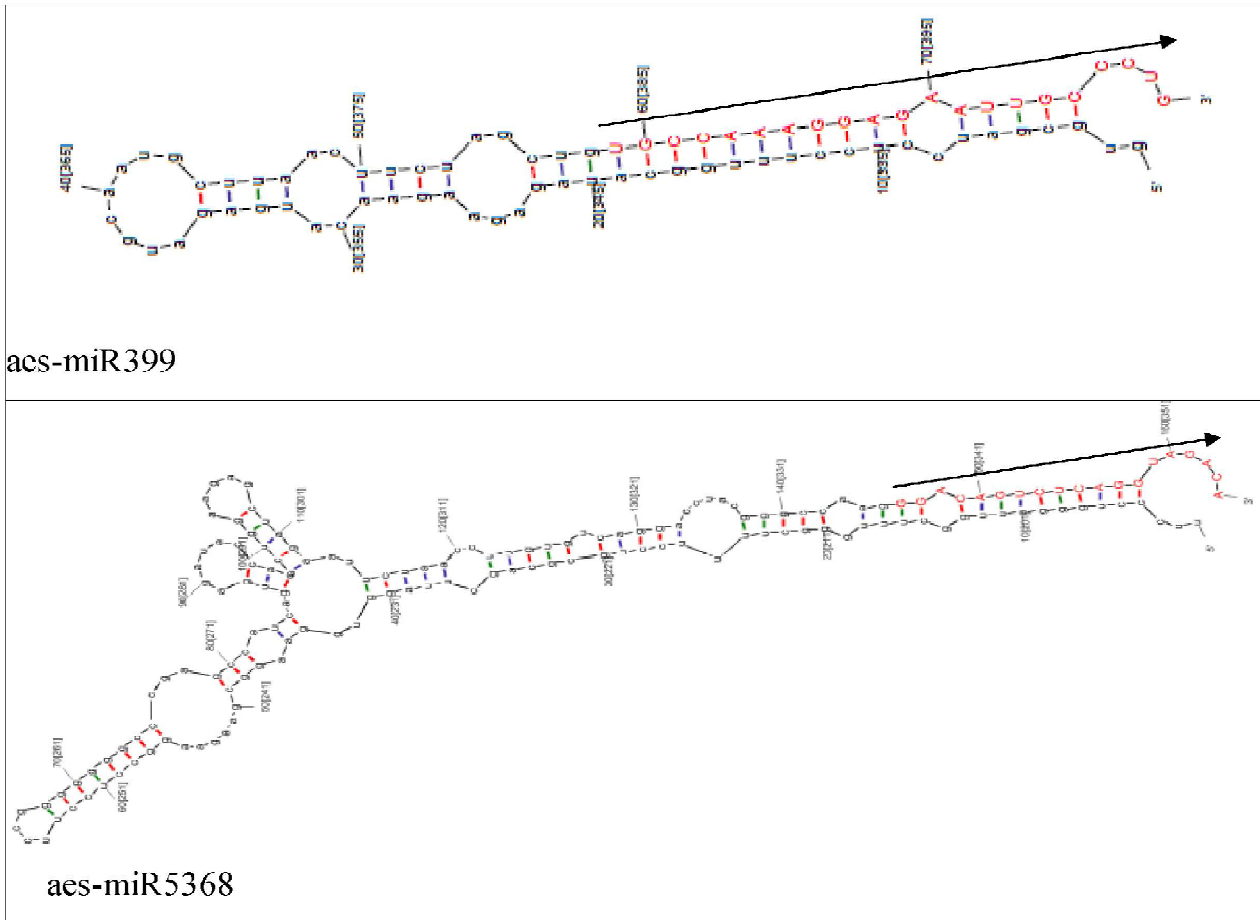


Fig 2: Putative secondary structure of new miRNA precursors and mature sequences identified in *Abelmoschus esculentus*. Blackarrows indicate the mature microRNA sequences.

Prediction of potential target genes and their functions

According to the previous studies, all miRNAs regulate gene expression by binding to the target mRNA sequence in a perfect or near-perfect complementary site (Bartel, 2004). By contrast in plants, miRNAs bind their targets by complete or nearly complete complementary sequences were as in animals miRNAs are partially complementary to their target mRNAs. For the target prediction analysis, there are different parameters involved which are shown to have the maximum expectation in the threshold of the score, and are responsible for the complementary between the miRNA (mainly miRNA and siRNA) and their target transcript. These alignments are responsible for the strong seed region complementarity, which is consecutive Watson-Crick matches on positions 2-7 at the 5' end of a miRNA (Krek *et al.*, 2005; Lewis *et al.*, 2005; Lim *et al.*, 2005).

The functional importance of the miRNAs identified here can only be understood when their respective target gene(s) are identified. It has been shown that one miRNA can target more than one regulatory gene (Bonnet *et al.*, 2004). First, many miRNAs directly targeted various transcription factors which control plant development and phase change from vegetative growth to reproductive growth. So, in order to understand the biological role of the particular miRNAs, target prediction are more important. Five target gene for aes-miR399 were predicted (**Table 2**). There are hundreds of miRNAs identified, were their majority of potential targets are unknown (Doench *et al.*, 2004). We were not able to find the targets for putative aes-miRNA5368 (Table 2). The function of MYB transcription factors play important roles in a variety of biological functions including plant development, hormone signaling and metabolism (Suo *et al.*, 2003).

Table 1. The predicted miRNAs in *Abelmoschus esculentus*, with MFE, MFEI and A+U content

Contig ID	Predicted miRNA	Predicted miRNA sequence	Strand	NM/nt	LM/nt	LP/nt	Location	(A+U) Content (%)	MFE (kcal mol ⁻¹)	MFEI
Contig738	aes-miR399	UGCCAAAGGAGA AUUGCCUG	+	1	21	78	3'	51%	31	-0.837
Contig198	aes-miR5368	GGACAGUCUCA GGUAGACA	+	1	19	163	3'	41%	58.5	-0.629

NM - number of mismatches, LM - length of mature miRNA, LP - length of pre-miRNA, NT -nucleotides, MFE - minimum free energy, MFEI - minimum free energy index

Table 2. The miRNAs and their putative targets genes with known functions in *Abelmoschus esculentus*.

miRNAs	Acc. no.	E-value	Alignment function	Regulated	Possible protein
aes-	TC264546	1.5	miRNA 20 UCCCGUUAAGAGGAAACCGU 1	Transcriptional regulatory protein	Regulation
	BF277561	3	miRNA 20 UCCCGUUAAGAGGAAACCGU 1 AGGGCUAUUCUUCUUUGACA 112	Target 93 Expressed protein	Unknown function
	TC239324	3	miRNA 20 UCCCGUUAAGAGGAAACCGU 1 Target 320 GGGGCAAUCAUCUUUGGCA 339	MYB transcription factor	Regulatory roles in developmental processes
	CO095104	3	miRNA 20 UCCCGUUAAGAGGAAACCGU 1 Target 231 AGGGAAAUUCUUCUUUGGU 250	ATP dependent Clp protease proteolytic	Stress response
	ES847673	3	miRNA 20 UCCCGUUAAGAGGAAACCGU 1 Target 29 AAGGAAAUUCUUCUUUGGCG 48	Triosephosphate isomerase	Metabolism
aes-miR5368No Targets Found					

Phylogenetic Analysis of miR399 family

The phylogenetic analysis of the miR399 family has revealed two major clusters (Fig 3). The newly predicted aes-miR399 was found in the same clade as that of mes-MIR399 (*Manihot esculenta*) which is also from Malvaceae family. The miR399 was found to be involved in the case of cis- regulatory element of endosperm expression (GCN₄-motif), MYB binding site involved in drought inducibility (MBS) and also in cis-acting regulatory element required for endosperm expression (Sk₁-motif) (Patanun et al., 2013). This indicates that our newly predicted aes-miR399 may be involved in drought inducibility/ endosperm expression.

A study has shown that approximately 10,000 ESTs in plants contain one miRNA (Zhang et al., 2006). According to this estimation 18 miRNAs should be predicted from 177,030 ESTs in *G. hirsutum* but they reported 30 miRNAs which means 1.67 miRNAs per 10,000 ESTs were predicted(Qiu.,et.al.2007). Similarly in *Vigna unguiculata* 20 miRNAs should be predicted from 2,41,854 ESTs whereas 47 were predicted, which depicts that just not only one gene might be regulated by individual miRNA but multiple miRNAs may target the same gene (Lu. Y et.al.2010). All these findings suggest that miRNA focus should be more on individual connections between miRNA and the target prediction. However, in the current study, we identified 2 miRNAs from 3,361 contigs which

were less in number when compared to the number of sequences in previous studies in other crops. In comparison to EST sequences, very less NGS sequences are required for prediction of miRNAs and their targets. Hence, this suggests that the efficient way for identifying miRNAs is through the EST analysis which would help in obtaining more miRNAs.

CONCLUSION

In this study, using bioinformatics approach, we were able to identify 2 miRNAs aes-miR399 and aes-miR5368 from NGS data of okra generated using the

454 Roche Titanium technology. For these two miRNAs, 5 target genes TC264546, BF277561, TC239324, CO095104 and ES847673 were identified in *A. esculentus*. The newly predicted target genes were involved in transcription factors, gene regulation, metabolism and developmental process. The result of this study will contribute to further research of miRNAs in the plant kingdom and their regulatory mechanisms in *Abelmoschus esculentus*. It can also be concluded that in comparison to EST sequences, very less NGS sequences are required for prediction of miRNAs and their targets.

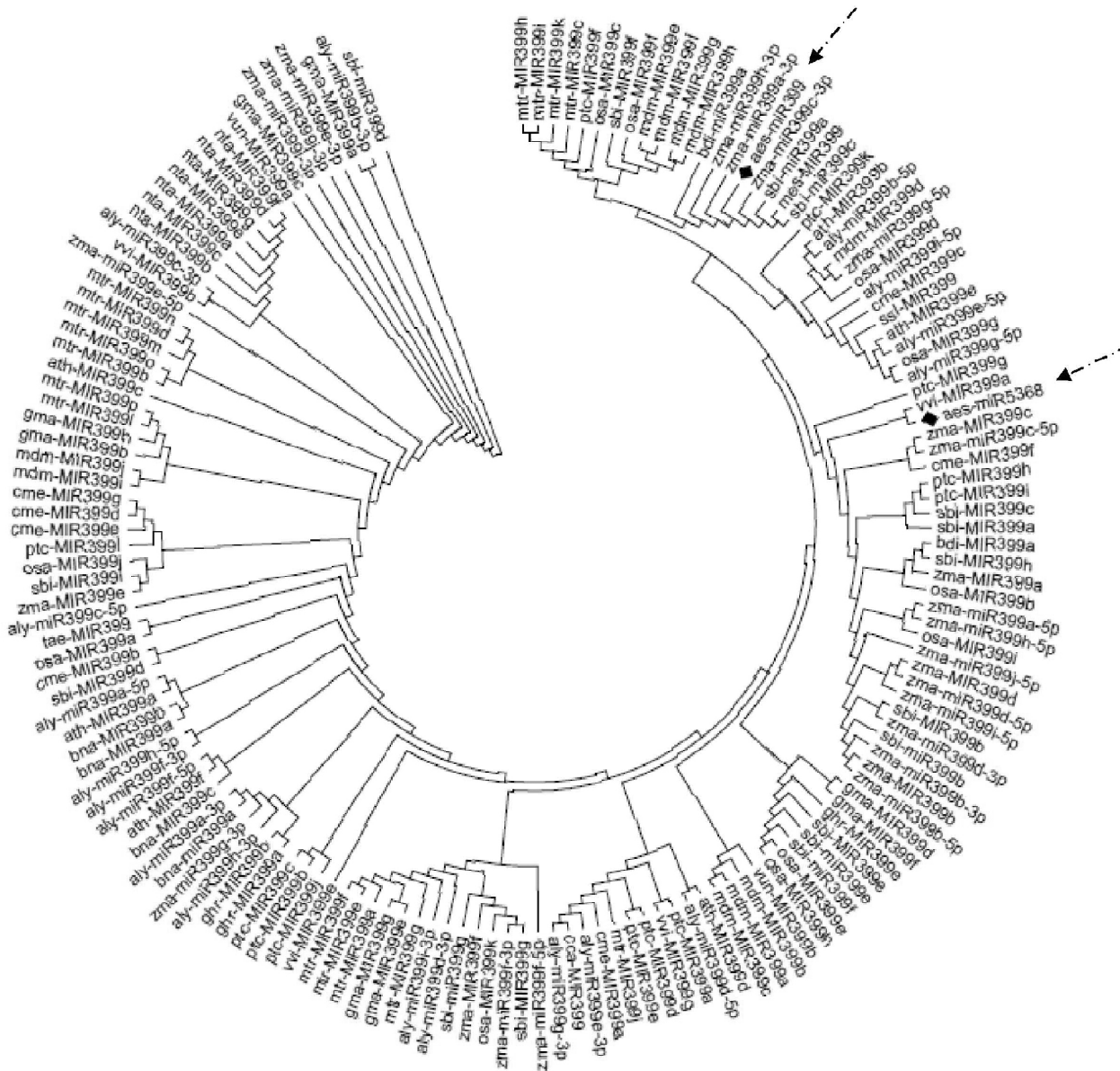


Fig 3: Phylogenetic tree of members of miR399 family

*The arrow (→) indicates the position of the newly discovered ‘aes-miR399’ and ‘aes-miR5368’ miRNA

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