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Research Article

Analysis of some conserved miRNAs in hazelnut (*Corylus avellena* L. and *Corylus colurna* L.) by real-time PCR

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ABSTRACT

Hazelnut is an important plant species which is used in food industry, dye industry, wood-chopping and stock farming and it has also benefits for health due to nutrient component. Economically valuable *Corylus avellena* and *Corylus colurna* used as rootstock are the most common cultivars. Although many studies have been made about microRNA in plants so far, there are few studies in hazelnut. miRNAs are 18–25 nucleotide, short and single strand non-coding RNAs. miRNAs called as post-transcriptional gene regulators cause repress or cleavage of their target mRNA. In particularly in plants, they cause cleavage of mRNA and so play role in developmental process, response process to biotic and abiotic stresses like drought, salt, cold or UV. Conserved miRNAs are miRNAs which have same function in different plant species and are conserved from very old times to the present. In this study, we aimed that analyzing of some conserved miRNAs (miR159, miR160, miR171, miR396, miR2919 and miR8123) in hazelnut (*Corylus avellena* L. and *Corylus colurna* L.) by Real-Time PCR. We found which these conserved miRNAs are present in both hazelnut species.

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INTRODUCTION

MicroRNAs are a group of non-coding small RNAs, that are 18–25 nucleotide in length [1]. According to the informations obtained from the miRBase database in 2019, novel miRNA analysis was performed for 271 organisms and 82 of them belong to the plant world. miRNAs can vary from species to species or may be tissue specific. These miRNAs are non-conserved. However, there are also conserved miRNAs in plant species. Conserved miRNAs are more than the non-conserved ones and are generally involved in developmental processes. By comparing the processes of

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Arabidopsis and maize miRNA, Willman and Poetig have demonstrated a similarity and a high degree of conservation between monocotyl and dicotyls [2].

In the post-transcriptional stage, miRNA binds to messenger RNAs (mRNAs). In plants, and rarely in mammals, perfect complementarity between the miRNA and the target mRNA induces the cleavage and disruption of the mRNA [3]. The microRNA genes are transcribed by RNA pol II in the nucleus, resulting in a hairpin-structured pri-miRNA primarily and then pri-miRNA is cleaved by the DCL1 enzyme to form the pre-miRNA. The pre-miRNA is converted into the microRNA duplex being further excised by the enzyme and then is exported to the cytoplasm. The miRNA is then incorporated in to the RNA-Induced Silencing Complex (RISC), which scans the cell for complementary mRNAs, with the guidance of fully or partially miRNA-mRNA complementarity. After base pairing occurs between the miRNA and its target mRNA, inhibition of translation or cleavage of target mRNAs are triggered within this complex [4].

Two hypotheses have been proposed regarding the evolutionary origins of miRNAs, inverted duplication hypothesis and transposable elements. According to the first hypothesis: MIR genes originate from inverted duplication of the target genes. When transcribed the inverted duplicated founder genes cause the emergence of proto MIRs. During evolution inverted repeats might shorten and mutate, resulting in the formation of pre-miRNA-like hairpins. The transcript of the founder gene serves as the target of the mature miRNA [5, 6]. Transposons, which are found in large numbers in many organisms are thought to be contributing to the origin and evolution of miRNA genes. Miniature inverted-repeat transposable elements (MITEs) a type of non-autonomous transposons, have the potential to be substrates for DCL proteins to generate miRNA genes [5].

All miRNAs are derived from hairpin transcripts and many are evolutionarily conserved. Therefore, identification of new miRNAs in different plant species is easier due to the presence of their orthologs and homologs [7]. However, unlike animals plants do not have precursors that are 70–80 nucleotides length. In plants, this number varies between 60 and 500, which makes it difficult to identify them in computational approaches [8]. Not only miRNA genes but also their target genes are conserved. Although there are several nucleotide changes between different types of targets, the sequences of the complementary regions are highly conserved. Floyd and Bowman [9] reported that HD-ZIP genes, the targets of miR-166, are conserved.

Conserved miRNAs have an important role in responding to environmental stimuli and in plant development processes. For example, miR159 targets GAMYB, a gene associated with the hormone gibberellin, causing late flowering [10]. mir160 has been reported to play a role in responding to the auxin signal by affecting the ARF genes, an auxin response factor [11]. Apart from these, miRNAs affect developmantal processes such as meristem cell identification, leaf and tissue morphogenesis, flower differentation and development, and stress responses [12, 13].

Hazelnut that is produced in Turkey, Italy, USA, Spain and some other countries has an important place in terms of human health and economic contribution [14]. Among the most widely grown hazels in Turkey, the most important species with the highest economic value are plump hazel (*Corylus avellena* L.) and Turkish hazel (*Corylus colurna* L.) which are used as rootstocks [15].

Besides its economic value, hazelnut is an important food source for human health and the food sector. In addition to being beneficial in reducing cancer and heart disease, it is widely used in the production of food products such as chocolate, cake and biscuits [16]. Since miRNAs play a curicial role in post transcriptional regulation of gene expression, important for plant growth and development, as well as plant stress responses, identifying conserved miRNA homologs in hazel species will provide an important contribution to both science and worl economy. The aim of this study is to determine the presence of 6 conserved miRNAs (miR-159, miR-160, miR-171, miR-396, miR-2919, and miR-8123) in *Corylus avellena* L. and *Corylus colurna* L. by using Real-Time PCR.

MATERIAL AND METHODS

Total RNA Isolation

The hazel tissues (leaf, bud, male and female flower) used in the study were obtained from Giresun Hazelnut Research Institute. Total RNA isolation was performed according to the protocol of miRCURY ™ RNA Isolation Kit (Exiqon). Quality and concentration of the isolatated total RNA were measured by Thermo Scientific Nanodrop One spectrophotometer.

Real Time PCR

miR-159, miR160, miR-171, miR-396, miR2919 and miR-8123 miRNAs, which were found to be conserved in plants in previous studies, were used for Real-Time PCR. Forward and universal reverse primer (Stratagene miRNA 1st-strand cDNA Synthesis Kit) sequences are shown in Table 1.

Poly-A Reaction: 4 µl of 5x poly-A buffer, 1 µl ATP (10 mM), 12 µl RNase-free water, 1 µl polymerase (E. coli PAP) and 2 µl of total RNA were mixed and total volume was completed to 20 µl. The reaction mixture was incubated first at 37 °C for 30 min and then at 95 °C for 5 min. cDNA Synthesis: 2 µl 10x Affinity buffer, 0.8 µl dNTP mix, 1 µl RT adapter primer, 1 µl RNase block enzyme mix, 4 µl poly-A reaction sample and 11.2 µl water were mixed. And then was incubated at 55 °C for 5 min, at 25 °C for 15 min, at 42 °C 30 min and at 95 °C 5 min, respectively. Real-Time PCR: 1 µl master cDNA samples, 12.5 µl master mix, 1 µl reverse primer, 0.33 µl forward primer and 25 µl of RNase-free water were mixed. For each miRNA, a no-template control was included to the reaction set up. The amplifica-

Table 1. Primer list: This table indicates primers that are used in Real-Time PCR analysis

Primers	Forward (5'-3')	Tm (°C)	Universal reverse (5'-3')	Tm (°C)
miR159	GTGGGTTTGGATTGAAGGG	57	GTGCAGGGTCCGAGG	56
miR160	TTGTGCCTGGCTCCCTGT	58	GTGCAGGGTCCGAGGT	56
miR171	GTTTTGATTGAGCCGTGCC	57	GTGCAGGGTCCGAGGT	56
miR396	GTGCAGGGTCCGAGGT	57	GTGCAGGGTCCGAGGT	56
miR2919	TTTTTTTCCCCCCCCCCC	58	GTGCAGGGTCCGAGGT	56
miR8123	GTTTGGGAACACGGTAAC	57	GTGCAGGGTCCGAGG	56

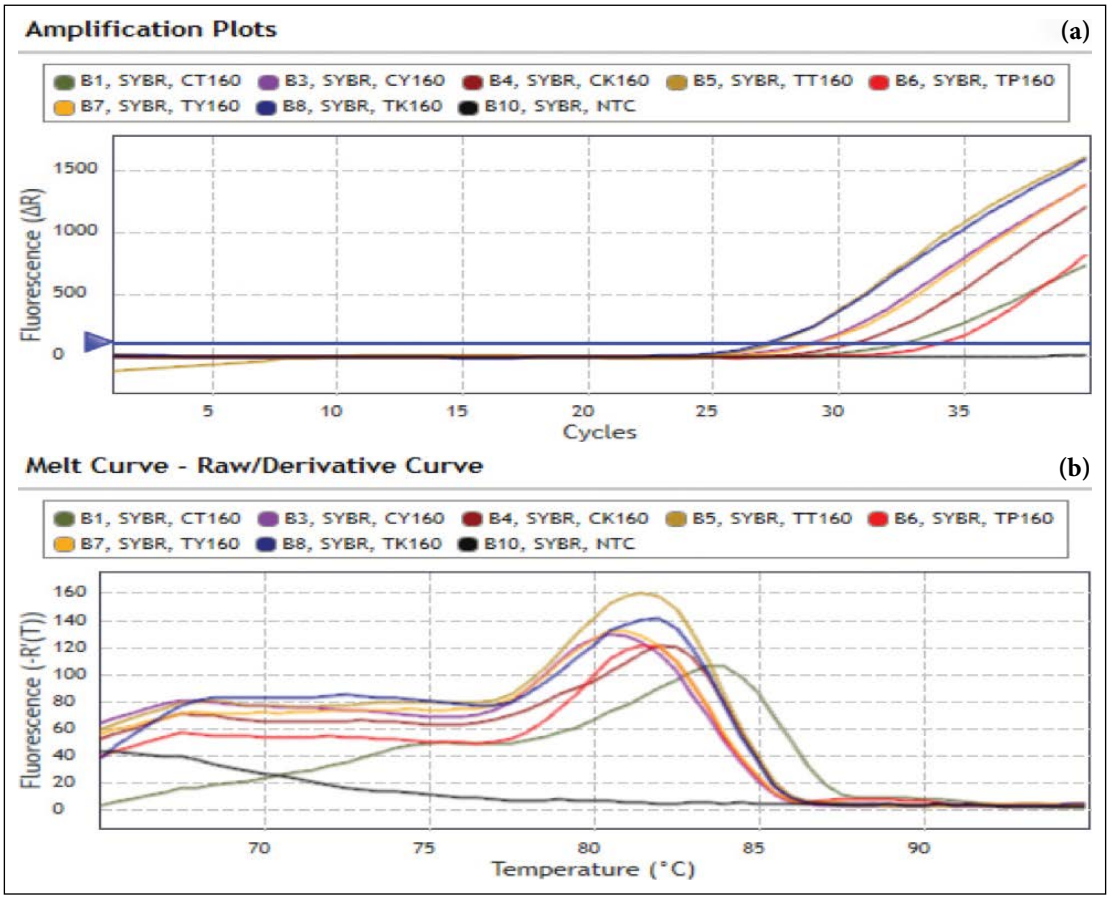

Figure 1. Real time PCR analysis of miR160. **(a)** This graphic indicates miR160 amplification in four tissues of *Corylus colurna* (T) and three tissues *Corylus avellena* (C). Green line: miR160 expression in bud tissue (T) of C, purple line: leaf tissue (Y) of C, dark red: female flower (K) of C. Orange line: bud tissue of T, red line: male flower of T, yellow line: leaf tissue of T and blue line: female tissue of T. Black one is NTC, shows whether there is contamination or non-desired product or not. **(b)** In this grapchic NTC has no peak so there is no contamination. Fluorescence (-R'(T)) is expression level.

tion was performend on AriaMx Real-Time PCR System (Agilent Technologies). Sequences of the specific primers for miR160, miR2919 and miR8123 used in the Real-Time PCR procedure are shown in Table 1.

RESULTS

miR160 detection in different tissue types of *Corylus avellena* and *Corylus colurna* by Real-Time PCR Analysis

The real- time PCR amplification and melt curve results for miR160 from *Corylus avellena* and *Corylus colurna* different tissues types are shown in Figure 1. We could detect the conserved miR160 in bud and female flower than other tissues in *Corylus avellena*. Cq values are 27,74 and 27,51 (Table 2). On the contrary, in bud of *Corylus colurna*, florescense value and Cq value of miR160 are 748,281 and 33,06 respectively. As a result, CT has the lowest amplification for miR160. In no-template control (NTC) has no peak. All tissues have amplification of miR160 (Fig. 1a, b). The highest mir159 amplification is in female flower of *Co-*

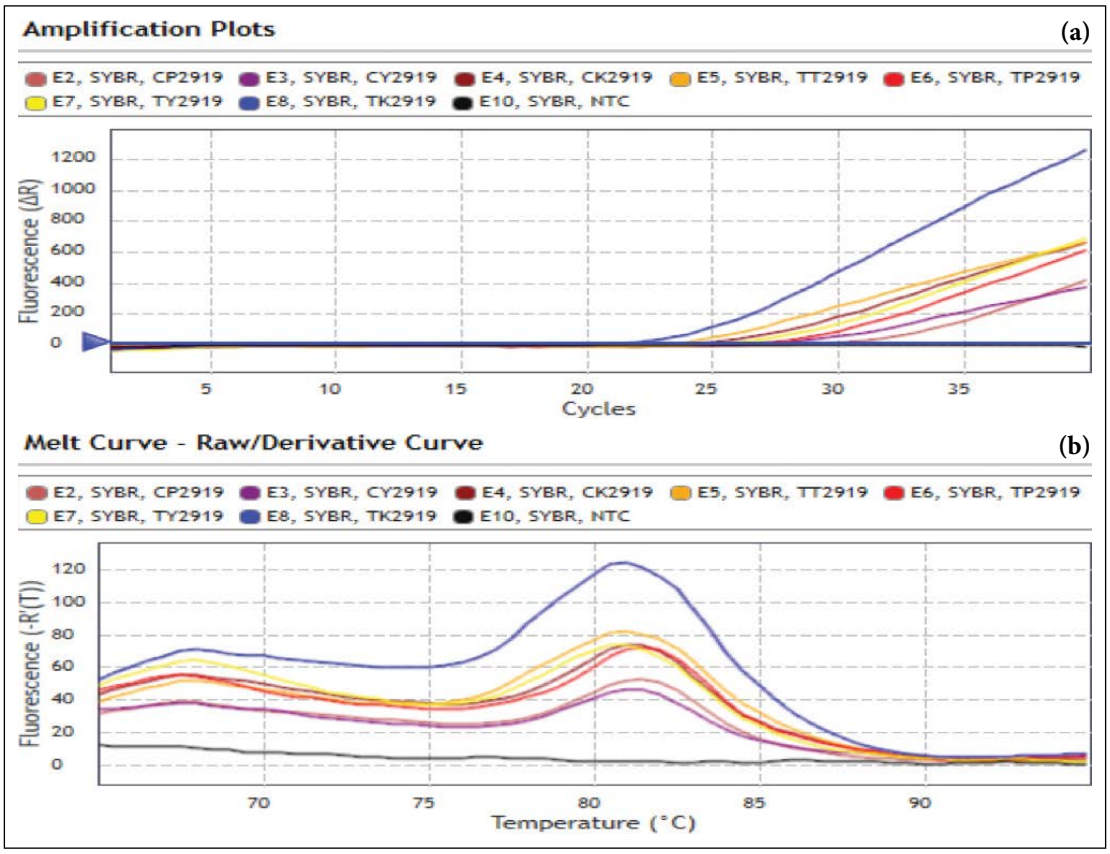

Figure 2. Real time PCR analysis of miR2919. **(a)** This graphic indicates miR2919 amplification in four tissue of *Corylus colurna* (T) and three tissues of *Corylus avellena* (C). The colurs represent the tissues and they are the same as figure 2. Pink line is male flower of *Corylus avellena*. Black one is NTC that shows whether there is contamination or non-desired product or not. **(b)** In this grapchic NTC has no peak so there is no contamination.

Table 2. The results of Real-Time PCR analysis for miR160. This table contains miR160 amplification based on ΔR Last, Cq and Tm Product. ΔR Last indicates fluorescence value of samples, parallel to amplification, and Cq value is cycle number which amplification starts. Tm is melting temperature of cDNA

Well name	ΔR last	Cq(ΔR)	Tm Product 1 (-R'(T))
CT160	748.28065	33.06	83.5
CY160	1407.4099	29.22	80.5
CK160	1215.9536	31.01	82
TT160	1620.3378	27.74	81.5
TP160	837.02475	34.44	81.5
TY160	1402.7008	29.51	80.5
TK160	1601.6224	27.51	82
NTC	10.37299	No Cq	65

rylus colurna (CK). Florescence value of CK159 is 2619,521 and Cq value is 21,04. The lowest mir159 amplification is in leaf tissue of *Corylus colurna* (CY) in compared to others. Florescence value of CY159 is 1823,794 and cq value is 21,58. Besides of these in other tissues and in two hazelnut species is seen amplification of miR159. (Data isn't shown)

The Lowest Amplified miRNA Among Other miRNAs is miR2919 in Real-Time PCR Analysis

In Real time data analysis, it is seen that miR2919 was lower expressed than other miRNAs. Additionally it amplified higher in female flower of *Corylus avellena* than other tissues but amplified the lowest in leaf tissue of *Corylus colurna* (Fig. 2a, Table 3). All tissues have only one peak in melting graphic (Fig. 2b). miR8123 is the lowest expressed miRNA following miR2919. Bud tissue of *Corylus avellena* has the highest miR8123 expression compared to others and male flower of *Corylus colurna* has the lowest miR8123 (Fig. 3a, Table 4). Because of low expression of miR8123 like miR2919, melting graphic is not clear. Only in leaf tissue of *Corylus avellena* are seen two peak but NTC is seen clear (Fig. 3b). Thus miR8123 has amplified slightly in the hazelnut.

DISCUSSION

To date, many studies have been conducted on miRNAs, which are involved in many molecular pathways

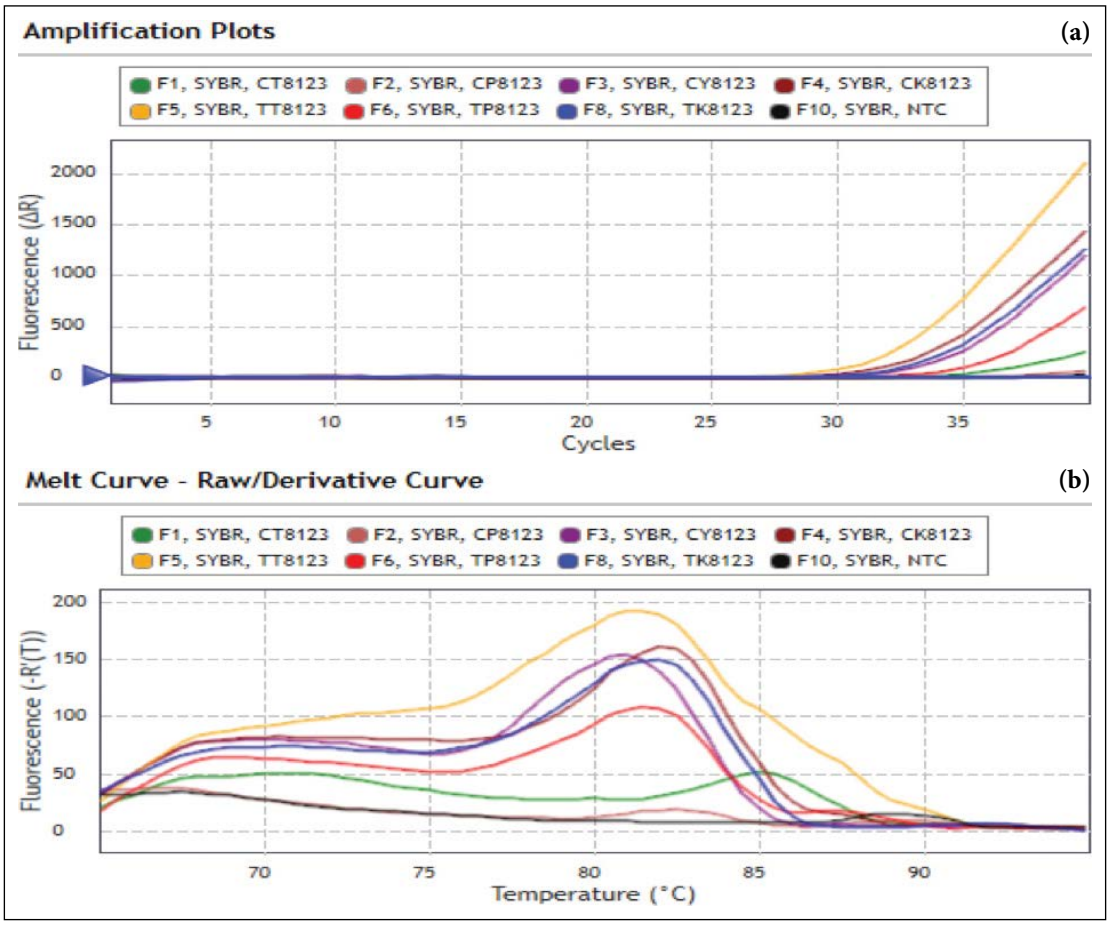

Figure 3. Real time PCR analysis of miR8123. **(a)** This graphic indicates miR8123 amplification in four tissue of *Corylus colurna* (T) and *Corylus avellena* (C). The colurs represent the tissues and they are the same as Figure 2. Black one is NTC that shows whether there is contamination or non-desired product or not. **(b)** In this grapchic NTC has no peak so there is no contamination.

Table 3. The results of real-time PCR analysis for miR2919: This table contains miR2919 amplification based on ΔR Last, Cq and Tm product

Well name	ΔR last	Cq(ΔR)	Tm Product 1 (-R'(T))
CP2919	429.73803	31.04	81.5
CY2919	378.73488	28.53	81.5
CK2919	666.29224	25.58	81.5
TT2919	669.53622	24.48	81
TP2919	621.974	27.84	81.5
TY2919	701.40086	26.67	81
TK2919	1269.06	22.47	81
NTC	-3.4880566	No Cq	65

Table 4. The results of real-time PCR analysis for miR8123. This table contains miR8123 amplification based on ΔR Last, Cq and Tm product

Well name	ΔR last	Cq(ΔR)	Tm Product 1 (-R'(T))
CT8123	250.5114749	35.07	85
CP8123	66.33285825	38.43	67
CY8123	1212.180384	31.59	81
CK8123	1454.020856	30.46	82
TT8123	2132.741574	28.93	81.5
TP8123	708.7195587	33.48	81.5
TK8123	1279.475748	31.24	82
NTC	29.33021137	No Cq	67.5

and have important roles in the post-transcriptional stage. miRNAs are responsible for degradation of mRNA in plants have been studied in various plant species such as poppy [17], wheat [18], cotton [19], peach [20], banana [21], corn [13], rice [22] and mostly Arabidopsis. In this study, it was aimed to detect some conserved miRNAs in two different hazelnut species by using Real-Time PCR.

miR160 was amplified earlier in the bud and female flower tissues of *Corylus avellena* than other tissues.

miR160 plays a role in response to auxin signaling and is also effective in leaf and root growth [11]. In the present study, the presence of this miRNA in the bud and the female organ suggests that it may also have a role in organ formation.

Previous studies have reported that in Papaver somniferum miR2919 is over-expressed in the capsule tissue and but lower in the leaf tissues [17]. In our study, miR2919 showed higher expression in the female flower of *Corylus avellena*, while it showed lower expression in the leaf tissue of *Corylus colurna*, parallel to previous poppy studies. According to this result, it can be suggested that miR2919 have a role in flower formation or development and leaf development [23]. miR8123 has been previously studied on potatoes and this miRNA has been shown to be expressed in underground shoots of potato [24]. According to our data, miR8123 showed the highest expression in the bud tissue of *Corylus avellena*. miR8123 is the least expressed in male flower tissue of *Corylus colurna* compared to other tissues.

The maximum proliferation for miR171 is seen in leaf tissue of both species. The florescence value of *Corylus avellena* leaf and *Corylus colurna* leaf is 3488,026 and 3207,895, respectively but NTC and melting graphic have extra peaks. Like miR171, different peaks are observed in the melting graphic of miR396. Although miR396 proliferates in tissues, these extra peaks are undesirable. (Data isn't shown)

In their study, Zhang et al. [25] developed a broad strategy by searching EST databases to identify new miRNA orthologs. They have reported that many miRNAs are conserved in species such as angiosperm, gymnosperm and algae. They identified 481 miRNAs belonging to 37 miRNA families from more than 6 million EST sequences, in 71 different plant species. They stated that miR-170/171 had orthologs in 22 different plant species. In addition to miR-171, they have found highly conserved miRNAs, including miR-159, miR-160, and miR-396. Willman and Poethig [2] compared two specific developmental miRNA pathways in Arabidopsis and maize and showed the level of similarity in these programs between a core eudicot and a monocot. As a result of these studies, it has been reported that some miRNAs are preserved in plant species, including gymnosperm, monocots and eudicots. The presence of orthologs of miRNAs in monocots and dicots indicates that miRNAs could exist more than 400 million years ago [25].

In this study, we showed that some of the known conserved miRNAs are also expressed in two different types of hazel spescies *Corylus avellena* L. and *Corylus colurna* L. To further verify these results the real time PCR products needs to be sequenced. The next step is to show the genes targeted by these miRNAs in bioinformatics-supported experiments with transcriptomic studies and to add new information to the miRNA literature.

AUTHORSHIP CONTRIBUTIONS

Concept: B.Y., N.Ö.Ö.; Design: N.Ö.Ö.; Supervision: N.Ö.Ö.; Data collection and/or processing: Z.Ö.U., S.U.; Analysis and/or interpretation: H.N.A., N.Ö.Ö.; Literature search: B.Y.; Writing: B.Y.; Critical review: N.Ö.Ö.

DATA AVAILABILITY STATEMENT

The published publication includes all graphics and data collected or developed during the study.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

REFERENCES

[1] Ambros V. microRNAs: Tiny Regulators with Great Potential. Cell Press 2001;107:823-826. https://doi.org/10.1016/S0092-8674(01)00616-X

[2] Willmann MR, Poethig, RS. Conservation and Evolution of miRNA Regulotary Programs in Plant Development. Science Direct 2007;10:503-511. doi:10.1016/j.pbi.2007.07.004.

[3] Axtell MJ, Bowman JL. Evolution of Plant MicroRNAs and Their Targets. Trends in Plant Science 2008;13:343-349. doi:10.1016/j.tplants.2008.03.009 A

[4] Eldem V, Okay S, Ünver T. Plant microRNAs: New Players in Functional Genomics. Turk J Agric For 2013:37:1-21. doi:10.3906/tar-1206-50

[5] Koptekin D. and Aktaş, L. Y., "Bitki MikroRNA'ları: Biyogenezi, Köken ve Evrimi", Türk Bilimsel Derlemeler Dergisi, 6: 140-148, 2013.

[6] Allen E, Xie Z, Gustafsa AM, Sung GH, Spatofora JW, Carrington JC. Evolution of MicroRNA Genes by Inverted Duplication of Target Genes Sequences in Arabidopsis thaliana. Nature Genetics 2004;36:1282-1290. doi:10.1038/ng1478

[7] Li A, Mao L. Evolution of Plant MicroRNA Gene Families. Cell Research 2007;17:212-218. doi: 10.1038/sj.cr.7310113

[8] Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanisims and Function. Cell 2004;116:281-29. doi: 10.1016/s0092-8674(04)00045-5

[9] Floyd SK, Bowman JL. Ancient microRNA Target Sequences in Plant. Nature 2004;428:485-486. doi:

10.1038/428485a

[10] Achard P, Herr A, Baulcombe DC, Harberd NP. Modulation of Floral Development by a Giberellin-Regulated MicroRNA. Development 2004;131: 3357-3365. doi: 10.1242/dev.01206

[11] Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, Chen XY. Control of Root Cap Formation by MicroRNA-Targeted Auxin Response Factors in Arabidopsis. The Plant Cell 2005;17:2204-2216. doi: 10.1105/tpc.105.033076

[12] Palatnik JE, Allen E, Wu X, Schammer C, Schwab R, Carrington JC, Wiegel D. Control of Leaf Morphogenesis by MicroRNAs. Nature 2013;425:257-263. doi:10.1038/nature01958

[13] Ding D, Zhang L, Wang H, Liu Z, Zhang Z, Zheng Y. Differential Expression of miRNAs in Response to Salt Stress in Maize Roots. Annals of Botany 2008;103:29-38. doi:10.1093/aob/mcn205

[14] T.C. Gümrük ve Ticaret Bakanlığı Kooperatifçilik Genel Müdürlüğü. 2016 Hazelnut Raport. Ankara: March 2017.

[15] Fındık Tanıtım Grubu, Fındık ve Sağlık, [Internet], http://www.ftg.org.tr/tr/turk-findigi-findik-ve-saglik.html, [October 25,2017].

[16] Karadeniz T, Bostan Z, Tuncer C. Tarakçıoğlu C. Fındık Yetiştiriciliği. Ziraat Odaları İl Koordinasyon Kurulu Yayınları. No 1, 2008, Ordu, 1545.

[17] Boke H, Ozhuner E, Turktaş M, Parmaksız I, Ozcan S, Unver T. Regulation of the Alkaloid Biosynthesis by miRNA in opium poppy. Plant Biotechnology 2015;409-420. doi: 10.1111/pbi.12346

[18] Yao Y, Guo G, Sunkar R, Du J, Zhu JK, Sun O. Cloning and Characterization of microRNAs from Wheat (Triticum aestivum L.). Genome Biology 2007;8: R96-R96.13. doi: 10.1186/gb-2007-8-6-r96

[19] Zhang B, Pan X. Expression of MicroRNAs in Cotton.Mol Biotechnol 2009;42:296-274. doi: 10.1007/s12033-009-9163-y

[20] Barakat A, Sriram A, Park J, Zhebentyayeva T, Main D, Abbott A. Genome Wide Identification of Chilling Responsive MicroRNAs in Prunus persica BMC Genomics 2012;13:481. doi: 10.1186/1471-2164-13-481

[21] Chai J, Feng R, Shi R, Ren M, Zhang Y, Wang J. Bioinformatic Identification and Expression Analysis of Banana MicroRNAs and Their Targets. Plos One 2014;10, e0123083. doi:10.1371.

[22] Sunkar R, Girke T, Jain PK, Zhu JK.Cloning and Characterization of microRNA from Rice. The Plant Cell 2005;17:1397-1411. doi: 10.1105/tpc.105.031682

[23] Baloch IA, Barozai MYK, Dın M. Identification and Characterization of 25 microRNAs and Their Targeted Proteins in Apricot (Prunus armeniaca L.). The Journal of Animal & Plants Science 2015;25:1466-1476.

[24] Dın M, Barozai MYK, Baloch IA. Identification anf Functional Analysis of New Conserved microRNAs and Their Targets in potato (Solanum tuberasum L.). Turkish Journal of Botany 2014; 38:1199-1213. doi: 103906/bot-1405-105.

[25] Zhang B, Pan X, Cannon CH, Copp GP, Anderson TA. Conservation and Divergence of Plant MicroRNA Genes. The Plant Journal 2006;46:243-259. doi: 10.1111/j.1365-313X.2006.02697.