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Molecular Characterization of *Elaeagnus angustifolia* L. Genotypes Collected from Different Parts of Turkey

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Abstract
Molecular markers are commonly used in determination of genetic similarities and differences in many species and varieties. In this research Inter-Simple Sequence Repeat (ISSR) markers were used to distinguish twenty-five elaeagnus (Elaeagnus angustifolia L.) genotypes which were collected from various parts of Turkey in accordance with specific morphologic criteria. Eleven ISSR primers produced a total of 92 fragments and 23 of them were polymorphic. The mean polymorphism information content (PIC) was 0.25. The unweighted pair group method arithmetic average (UPGMA) analysis demonstrated that the accessions had a similarity range from 0.63 to 1.00. Relatively genetic variation was detected among genotypes. Apart from the two genotypes, all other genotypes are separated. 'Genotype 72' is the most distant genotype. The remaining 24 genotypes were collected under three main groups. On the other hand, some of the genotypes are grouped according to their geographical distribution. The study showed that there is variation among genetic resources and that could be used in breeding programmes.

Keywords: Elaeagnus angustifolia L., molecular characterization, ISSR

INTRODUCTION

Elaeagnus angustifolia L. belongs to the genus *Elaeagnus* of *Elaeagnacea* family and the family comparises three genera: *Elaeagnus* L., *Hippophae* L., *Shepherdia* and has seventy seven species worldwide [1] and [2]. *Elaeagnus angustifolia*, commonly called wild olive, silver berry, Russian olive or oleaster, native to central and western Asia, Afghanistan, from southern Russia and Kazakhstan to Turkey, parts of Pakistan and Iran. It is now also widely established in North America as an introduced species [3]. In Turkey, the tree grows up to two thousand meter of altitude, around the Mediterranean sea, Black Sea Region, Marmara Sea Region and East Anatolia Region [4].

E. angustifolia is a fast-growing tree, grows up to 10 m in height and its trunk is up to 30 cm in diameter . Its crown is patulous, with reddish brown or silvery branches having spines about 3 cm long [5]. It is classified sometimes a shrub or a small tree. Its flowers are yellowish white, have very sharp and beautiful fragrance. The fruit ellipsoid or subglobose, within the fruit, there is a single seed oval or pointed, of five to ten mm length [2]. Fruit and leaf image of this species provided in Figure 1.

The ability of *E. angustifolia* to establish growth made it naturalized, has invaded zones along watercourses in many arid and semiarid regions of the world [6] and [7]. It was reported that wild olive plays a very important role in maintaining ecosystem functions in hyperarid areas because of its tolerance to severe drought, high alkalinity and salinity, in soils [8] and [9]. The species is also often used in agricultural settings, playing a significant role in carbon sequestration and natural resource conservation[10].

The *E. angustifolia* species has also various medicinal and economical uses. The ripe fruits of it have been used to treat amoebic dysentery. Wild olive fruit or flower preparations are used for treating vomiting, nausea, jaundice, flatulence and asthma in folk medicine [8] and [9]. Fruits and leaves of *E. angustifolia* have antipyretic effect.There is a common belief that fruits and leaves of *E. angustifolia* have antipyretic effect [11]. An infusion of the fruit has been used in Iranian traditional medicine as an analgesic agent for alleviating pain in rheumatoid arthritis patients. The flower is also traditionally used for treating tetanus [12; 13].

Figure 1. Fruit and leaf color and shape of *E. angustifolia.*

For the characterization of genetic resources in plants morphological, physiological and cytological markers were initially used. Later, biochemical markers were improved to further shorten and strengthen this stage. In recent times, molecular studies have gained acceleration [14].

Molecular markers display nucleic acid sequence differences in DNA in the cells of the plants in which they are produced in various forms. They are evaluated with close to 100% confidence in the diversity in the plant population or in the relationships among plant genotypes within that population. The aim in DNA markers is to reveal the difference in DNA level among individuals (variety, line,

species, etc.). If this difference shows a single region in the genome, it is called an allele. The main advantage of doing this at the DNA level is that any DNA chain can show allelic variability between two individuals. For this, it is not necessary to know whether that DNA sequence encodes any protein. There are several factors that affect the marker system to be used. Polymorphism level or type of population, stability in different environments, number of loci, ease, cost of analysis, infrastructure are some of these criteria [15].

Basically, two different DNA marker techniques are available. The use of DNA markers in plants has started with non-PCR (Polymerase Chain Reaction)-based RFLP (Restriction Fragment Length Polymorphism) markers, and PCR-based DNA markers; SSR (Simple Sequence Repeats), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and SRAP (Sequence Related Amplified Polymorphism) with the emergence of PCR technology; has begun to be used in genetic definitions [16].

Identifying the genetic diversity, in phylogenetic studies, formation genomic maps and in evolutionary biologyis, ISSR is an effective technique that can be applied to many plants [17]. This is a method based on random distribution of nucleotide units such as two, three, four, five repeating in eukaryotic genomes, which is sensitive and highly reproducible, in a locus independent manner in the genome therefore stands out among other methods [18] and [19]. Since ISSR markers are easy to apply and have longer primers, they are more reliable and faster to use [20]. Using ISSR primers that provide sufficient information, time savings, low cost and practicality in genetic analysis [21].

To the best of our knowledge, the genetic diversity of *E. angustifolia* has been analyzed based on RAPD molecular markers and morphological traits [22] and ISSR markers [9] and there is only a few published report using DNA marker system in Turkey's *E. angustifolia* germplasm. Our work, will give preliminary information about the genetic diversity of this plant in order to protect the *Elaeagnus angustifolia* germplasm in Turkey and for use it in breeding programs.

MATERIALS AND METHODS

Plant materials

Twenty-five *E. angustifolia* accessions from various districts and altitudes were collected from 7 different regions of Turkey; Nevsehir (Genotypes 1-5, 25), Aksaray (Genotypes 6, 7, 12-14), Konya (Genotypes 8-10), Sivas (Genotypes 15-17), Malatya (Genotypes 18-20), Adiyaman (Genotypes 21-23) and Kahramanmaras (Genotypes 24) were used as the plant material for the present study.

DNA extraction

Total genomic DNA was extracted from fresh young leaves following the cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1987) with minor modifications [23]. The concentration of each DNA bulk sample was determined spectrophotometrically at 260 nm (BioTek Instruments, Inc., Winooski, VT, United States). The quality of the DNA was checked by running 1 µl DNA in 1% (w/v) gels in TE solution (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). DNA samples that gave a smear in the gel were rejected. DNA was diluted to make uniform concentration of 10 ng/µl for PCRs.

ISSR amplification

PCR amplifications were performed according to the protocol of Uzun et al. (2009) [24]. ISSR amplification reactions were carried out in 15 µl volume containing 1.5 µl MgCl2 (50 mM), 0.33 µl dNTP (10 mM), 1.5 µl PCR buffer (10x), 0.20 µl Taq DNA polymerase (5u / µl), 1.5 µl Primer (100 µM), 2 µl template DNA (10ng/ µl) and 7.97 µl ddH2O. A total of 18 ISSR primers were used for all genotypes and 11 gave distinct polymorphic products (Table 1). DNA amplifications were performed using a DNA thermal cycler (Sensoquest Progen Scientifc Ltd., Mexborough, South Yorkshire, UK). The amplification reactions were carried out using Time Release program following these steps: Initial denaturation for 3 min at 94 ºC, followed by 35 cycles of 94 ºC for 1 min, annealing at 53 ºC for 50 sec, extension at 72 ºC for 2 min 2 repeats and a final extension at 72 ºC for 7 min. Tubes were held at 4 ºC until removal.

Electrophoresis

Amplified products were electrophoresed (Cleaver Scientific, Major Science) on 2% agarose gel with 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 110 V for 2.5–3 h. The gels stained with ethidium bromide (1.0 µg/ml). The fragment patterns were photographed under UV light for further analysis. A 100-bp standard DNA ladder was used in both analyses as the molecular standard in order to confirm the appropriate markers. Primers which gave reproducible fingerprints (DNA bands) were considered for the data analysis.

Data analysis

Each ISSR band was considered as an independent locus and polymorphic bands were scored as absent (0) or present (1) for all the 25 genotypes. Only clearly reproducible bands were scored and differences in band intensity were not considered. Faint or unclear bands were not considered. Unweighted Pair Group Method with Arithmetic Averages (UPGMA), and dendrograms were constructed using NTSYS pc 2.11 sofware [25]. The genetic relationships among the genotypes were examined using UPGMA cluster analysis through Nei's pairwise genetic distance.

RESULTS AND DISCUSSION

ISSR amplifcation

Size of DNA fragments were estimated by comparison with the DNA size marker 100 to 3000 bp. A total of 18 primers tested, 11 gave distinct polymorphic products (Table 1). Typical results obtained with primer VHV$(GTG)_7$ are shown in Figure 2. The size of the fragments ranged from 50 to 1,500 bp. The number of bands scored per primer for the ISSR analysis ranged from 5 ((AG)8T, (GACA)4, (GT)8YA and $(CA)_8$R) to 11 ($(AG)_6$GC, $(CAC)_3$G and HVH$(CA)_7$T), with a mean of 8.36.

Table 1. List of ISSR primers used in the study, and their fragment lenght (FL), total fragment number (TFN), Polymorphic fragment number (PFN) and percentage of polymorphism (PP)

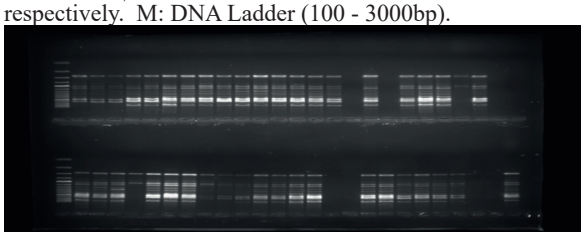
Primer	FL (bp)	TFN	PFN	PP (%)
$(AG)_8$T	500-1500	5	1	20.0
$(GACA)_4$	475-1100	5	2	40.0
$(AG)_7$YC	50-450	10	0	0.0
$(GT)_8$YA	390-1400	5	0	0.0
HVH$(CA)_7$T	200-1000	11	3	54.5
BDB$(CA)_7$C	140-1100	10	6	60.0
$(AGC)_6$G	200-1100	11	2	18.1
$(CAC)_6$	350-1400	9	7	77.7
$(CA)_8$R	850-1500	5	0	0.0
VHV$(GTG)_7$	275-1400	10	1	10.0
$(CAC)_3$G	350-1000	11	1	9.09
Mean	-	**8.36**	**2.09**	**25.0**
Total	-	**92**	**23**	**-**

The number of polymorphic fragments varied between 0 ((AG)7YC, (GT)8YA and $(CA)_8R$) and 7 ($(CAC)_6$), with a mean of 2.09. Eleven primers generated 92 scorable fragment, which 23 were polymorphic (25 %). Asadiar et al. (2012) [22], obtained 8.3 polymorphic fragments per primer and found a polymorphism rate of 79%. Uzun et al. (2015) [26], obtained 7.66 polymorphic fragments per primer and found a polymorphism rate of 81.92%. The differences among the studies may be caused by geographic factors, a high level of worldwide diversity within *Elaeagnus* spp.the limited area of the studies.

Genetic similarity analysis

The data of the ISSR analyses were used to perform a genetic diversity analysis among the 25 *E. angustifolia* accessions. The unweighted pair group method arithmetic average (UPGMA) analysis demonstrated that the accessions had a similarity range from 0.63 to 1.00 (Figure 3). Relatively genetic variation was detected among genotypes. *E. Angustifolia* was propagated by seed and vegatative sections. So, obtained relatively genetic variation may be expalined by vegatative propagation of this plant. Apart from the two genotypes (genotypes 6 and 21), all other genotypes are separated. 'Genotype 22' which was collected from Adiyaman, Turkey, is the most distant genotype. The remaining 24 genotypes were collected under three main groups. The firs group consisted of genotypes 15, 16,17, 18, 19, 23 and 24. These genotypes collected from closely related region of Turkey that Sivas, Malatya, Adıyaman and Kahramanmaras provinces. Ten genotypes (6, 7, 8, 9, 10, 11, 12, 13, 14, 21) nested in group 2. All genotypes except number 21, collected from Aksaray and Konya provinces which was in same region of Turkey. The last group of dendrogram was consisted of seven genotypes (1, 2, 3, 4, 5, 20, 25). In this group, except genotype 20, all of others were taken from Nevsehir province. In general, genotypes were grouped according to their geographical distribution. The genetic similarity of 9 Iranian *E. angustifolia* genotypes was determined to be between 0.51 and 0.77, according to ISSR data [22]. On the other hand the genetic distance of 56 *E. angustifolia* accessions collected from the Central Anatolian region of Turkey was between 0.00 and 0.34 [26].

Figure 2. Amplified PCR products generated using primer $VHV(GTG)_7$ for 25 genotypes of *E. angustifolia.*1-25 respectively. M: DNA Ladder (100 - 3000bp).



CONCLUSION

Our research is the one of the prominent evaluation of genetic diversity of *E. angustifolia* in Turkey and the study showed that there is relatively variation among genetic resources. Regarding to results of this study, more DNA markers should be used for diversity analysis of this species.

In conclusion, there is a high genetic variability among studied *E. angustifolia* genotypes in Turkey. This present study showes that PCR based fingerprinting techniques (ISSR) are informative for estimating the extent of genetic diversity as well as determining the pattern of genetic relationships and that could be used in breeding programmes.

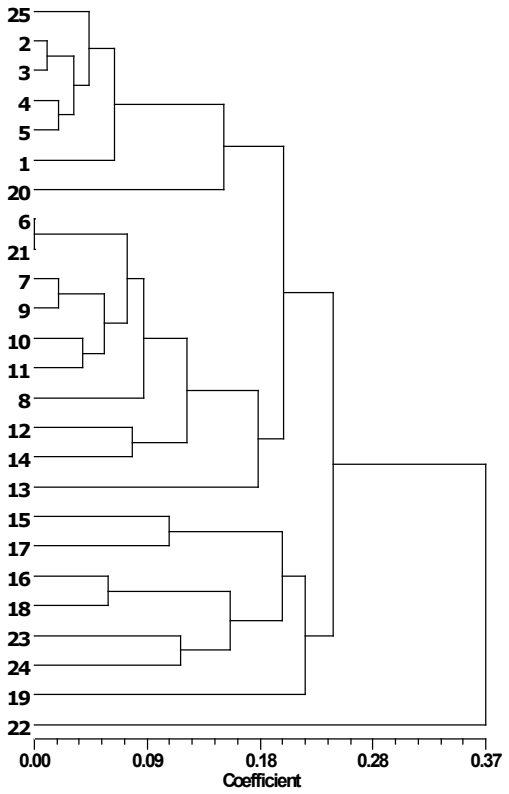


Figure 3. UPGMA dendrogram of the 25 *E. angustifolia* genotypes based on Nei's genetic distance and ISSR data. Codes of genotypes represent in the Material section.

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