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Assessment of genetic diversity in the Russian olive *(Elaeagnus angustifolia*) based on ISSR genetic markers[1]

Avaliação da diversidade genética em Oliva Russa (*Elaeagnus angustifolia*) com base em marcadores genéticos ISSR

Leila Sadat Asadiar[2]*, Fatemeh Rahmani[2] e Abbas Siami[2]

ABSTRACT - *Elaeagnus* is a Eurasian tree with 77 species worldwide. In this study, ISSR markers were used to establish the level of genetic relationships and polymorphism across nine genotypes of *Elaeagnus angustifolia* collected from 9 different regions of West Azarbaijan province. The ISSR analysis with 11 anchored primers also generated 116 scorable loci, of which 92 were polymorphic (79.3%). The estimated Jaccard similarity coefficient ranged from 0.44 to 0.76 for the ISSR markers. Cluster analysis was carried out, based on the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and the dendrogram drawn with the help of the NTSYSpc 2.02 software. The analysis revealed 5 main clusters for the ISSR data. According to our results, there is a relatively high genetic distance across *E. angustifolia* genotypes in the West Azarbaijan province of Iran. Furthermore, it could be inferred that ISSR markers are suitable tools for the evaluation of genetic diversity and relationships within the *Elaeagnus* genus.

Key words: Dendrogram. *Russian olive*. Genetic variation. Molecular marker.

RESUMO - A E*laeagnus* é uma árvore da Eurásia com 77 espécies em todo o mundo. Neste estudo, marcadores ISSR foram usados para estabelecer o nível de relações genéticas e polimorfismo entre nove genótipos de *Elaeagnus angustifolia*, coletados em 9 diferentes regiões da província do Azerbaijão Ocidental. A análise ISSR, com 11 primers ancorados, também gerou 116 loci contáveis, dos quais 92 polimórficos (79,3%). O coeficiente de similaridade de Jaccard estimado, variou de 0,44 a 0,76 para os marcadores ISSR. A análise de agrupamento foi realizada com base no Método não-ponderado de pares não-agrupados, com médias aritméticas (UPGMA), e a dendrograma elaborada com a ajuda do software NTSYSpc 2.02. A análise revelou cinco grupos principais para os dados ISSR. De acordo com nossos resultados, há uma distância genética relativamente alta entre genótipos de *E. angustifolia* na província de Azarbaijan Ocidental no Iran. Além disso, pode-se inferir que os marcadores ISSR são ferramentas adequadas para a avaliação da diversidade genética e as relações dentro do gênero *Elaeagnus*.

Palavras-chave: Dendrograma. Oliva russa. Variação genética. Marcador molecular.

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INTRODUCTION

The Elaeagnaceae is a small family with three genera: *Elaeagnus L.*, *Hippophae L.* and *Shepherdia Nutt* and has 77 species worldwide (SUN; LIN, 2010). Temperate Eurasian *Hippophae* as well as North American *Shepherdia* only consists of three species, whereas *Elaeagnus L.* is the largest with a wide distribution in eastern Asia, extending to Southeast Asia and Queensland in northeastern Australia. The diversity of the genus *Elaeagnus* is centered in China (SUN; LIN, 2010). *E. angustifolia* is a deciduous tree, sometime shrub, erect, to 10 m tall in cultivation. Flowers are yellowish white and fruit ellipsoid or subglobose (SUN; LIN, 2010).

This Eurasian tree has become naturalized and has invaded zones along watercourses in many arid and semiarid regions of the world (KLICH, 2000). *E. angustifolia* plays a very important role in maintaining ecosystem function in the hyper arid areas, because of its tolerance to severe drought, high salinity and alkalinity in soils (WANG *et al*., 2006). The species has various ecological, medicinal and economical uses. The ripe fruits of *E. angustifolia* have been used to treat amoebic dysentery. In folk medicine, oleaster fruit or flower preparations are used for treating nausea, vomiting, jaundice, asthma and flatulence (WANG *et al.*, 2006). 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 traditionally used for treating tetanus (HOSSEINZADEH; RAMEZANI; NAMJO, 2003).

Although morphological traits are valuable in identifying genotypes, the results are not completely reliable because morphological traits can be affected by environmental factors and cultivation conditions (STRUSS *et al*., 2001). These markers directly measure variations at the DNA level and are not affected by environmental factors and developmental stages (SARABI *et al*., 2010; SARKHOSH *et al*., 2006).

Since the mid 1980s, PCR (Polymerase Chain Reaction) technology and a large number of marker protocols have been developed. PCR based techniques developed in recent years such as Random Amplified Polymorphic DNA (RAPDs) (WELSH; MCCLELLAND, 1990; WILLIAMS *et al*., 1990), Inter Simple Sequence Repeats (ISSR) (ZIETKIEWICZ; RAFALSKI; LABUDA, 1994), Amplified Fragment Length Polymorphism (AFLPs) (VOS et al., 1995) and Simple Sequence Repeats (SSR) (WEBER; MAY, 1989).

All these DNA markers are dispersed throughout plant genomes and are easier to reproduce and analyze. Since 1994, inter-simple sequence repeat (ISSR) molecular marker technique developed by Zietkiewicz, Rafalski and Labuda (1994) has become available. ISSR markers have the advantages of high reproducibility and relatively low cost (GONZALEZ; COULSON; BRETTELL, 2002; GOULAO; OLIVEIRA, 2001).

ISSR markers can also be applied in population genetic studies of plant species as they effectively detect very low levels of genetic variation (ZIETKIEWICZ; RAFALSKI; LABUDA, 1994). They also require small amounts of DNA without involving radioactive labels and no prior genomic information is required for their use (BORNET; BRANCHARD, 2001).

Based on literature research, genetic relationship of *E. angustifolia* has been analyzed based on RP-HPLC biochemical markers (WANG *et al*., 2006) and there is no published report using any DNA marker system in the world. In the present study, we employed ISSR marker to investigate genetic variation among different genotypes of *E. angustifolia*.

MATERIAL AND METHODS

Plant material

Leaves of *E. angustifolia* were collected from 9 different regions of West Azarbaijan province of Iran in September 2010 and transferred to -80 °C freezer in Biotechnology Research Center of Urmia University (Tabela 1 and Figura 1).

DNA extraction

Total genomic DNA was extracted from fresh young leaves taken from 5 plants per location following the cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1987) with minor modifications. The concentration of each DNA bulk sample was determined spectrophotometrically at 260 nm (BioPhotometer 6131; Eppendrof, Hamburg, Germany). The quality of the DNA was checked by running 1 µl DNA in 1% (w/v) gels in 0.5% TBE buffer (45 mM Tris base, 45 mM boric acid, 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.

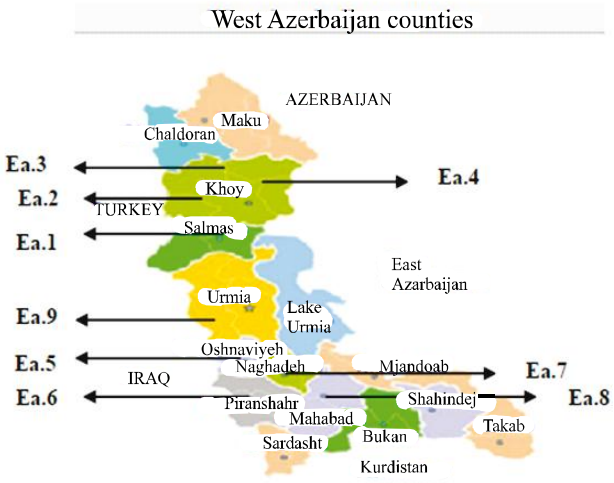
ISSR amplification

PCR amplification was performed according to the protocol of Zietkiewicz, Rafalski and Labuda (1994). ISSR amplification reactions were carried out in 25 µl volume containing 0.75 µl MgCl2 (50 mM), 0.5 µl dNTP (10 mM), 2.5 µl PCR buffer (10x), 0.25 µl Taq DNA

Table 1 - Location of *E. angustifolia* genotypes collected from West Azarbaijan province of Iran

Genotype	Code	Location	Habitat (m)	Latitude (E)	Longitude (N)
1	E.a1	Salmas	1,394	38˚ 13’	44˚ 48’
2	E.a2	Road ghotor khoy	1,170	38˚ 3´	44˚ 57’
3	E.a3	Road hamzian khoy	1,129	38˚ 35’	44˚ 57’
4	E.a4	Road maku khoy	1,075	38˚ 34’	45˚ 03’
5	E.a5	Oshnaviyeh	1,505	37˚ 02’	45˚ 05’
6	E.a6	Piranshahr	1,510	36˚ 42’	45˚ 08’
7	E.a7	Naghadeh	1,332	36˚ 56’	45˚ 23’
8	E.a8	Mahabad	1,395	36˚ 44’	45˚ 41’
9	E.a9	Urmia (Band)	1,404	37˚ 30’	45˚ 00’

Figure 1 - Geographical distribution of selected *E. angustifolia* genotypes in West Azarbaijan province as has been described in Table 1



polymerase (5u / µl), 0.3 µl Primer (100 µM), 1 µl template DNA (10ng/ µl) and 19.7 µl ddH2O. Eighteen ISSR primers were purchased from Cinnagen, Inc., Tehran, Iran (Tabela 2). DNA amplifications were performed using Veriti ® 96 well Thermal Cycler (Applied Biosystem, USA). The amplification reactions were carried out using Time Release program following these steps: Initial denaturation for 3 min at 95 ºC, followed by 35 cycles of 95 ºC for 30 sec, annealing at 50 ˚C for 45 sec, extension at 72 ºC for 2 min and a final extension at 72 ºC for 10 min. Tubes were held at 4 ºC until removal.

Electrophoresis

Amplified products were electrophoresed (Bio-Red Company, Eppendorf) on 2% agarose in 0.5X TBE buffer. The gels stained with ethidium bromide (1.0 µg/ml) and photographed under UV light in a gel documentation system (Carestream 212 Pro, USA). Primers which gave reproducible fingerprints (DNA bands) were considered for the data analysis.

Data analysis

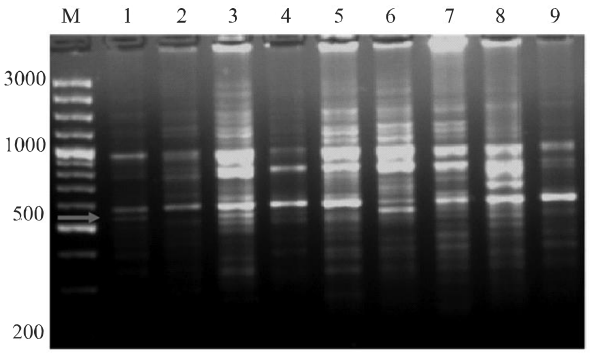
Each ISSR band was considered as an independent locus and polymorphic bands were scored as absent (0) or present (1) for all the 9 genotypes. Only clearly reproducible bands were scored and differences in band intensity were not considered. Faint or unclear bands were not considered. Data analyses were conducted using only the polymorphic bands. Genetic similarity matrix was generated using Jaccard’s similarity coefficient (JACCARD, 1908). The cluster analysis was performed based on Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and dendrogram drawn by help of NTSYS pc 2.02 software (ROHLF, 2000).

RESULTS AND DISCUSSION

Out of 18 ISSR primers tested, 11 gave distinct polymorphic products. Typical results obtained with primer 825 are shown in Figure 2.

The size of the amplified products ranged from 200 to 3,000 bp. Eleven primers generated 116 scorable loci, which 92 were polymorphic (79.3%). The number of bands varied from 4 (primer 868) to 19 (primer 826) with an average of 10.5 markers per primer. The polymorphism level, calculated as the number of polymorphic bands per primer ranged from 25% (primer 868) to 100% (primers 826, 848). The average number of polymorphic bands per primer was 12.6. Minimum and Maximum number of polymorphic bands was obtained with the primers 868 (1) and 826 (19), respectively (Tabela 2). Two primers showed high level of polymorphism, seven revealed relatively

Figure 2 - Amplified PCR products generated using primer 825 for 9 genotypes of *E. angustifolia*.1, 2, 3, 4, 5, 6, 7, 8, 9 are representative of the Salmas (E.a1), Jade ghotor khoy (E.a2), Jade hamzian khoy (E.a3), Jade maku khoy (E.a4), Oshnaviyeh (E.a5), Piranshahr (E.a6), Naghadeh (E.a7), Mahabad (E.a8) and Band (E.a9), respectively. M: DNA Ladder (100 - 3000bp). The arrow Shows polymorphic band



informative (≥ 50%) and two were comparatively less informative (< 50% polymorphism).

The similarity coefficient among different genotypes of *E. angustifolia* ranged from 0.44 (between E.a2 and E.a4) to 0.76 (between E.a6 and E.a8) (Tabela 3).

The cophenetic correlation coefficient between the similarity matrix and the UPGMA dendrogram was r = 0.8, indicating a relatively good representation of relationships among genotypes in the dendrogram. The dendrogram revealed five main clusters (Figura 3).

Cluster I consisted of E.a1 and E.a4. Cluster II was further divided into 2 sub clusters. The first sub cluster consisted of E.a3 and the second sub cluster comprised of E.a6 and E.a8. Clusters III and V each included one genotype, E.a2 and E.a9, respectively. E.a5 and E.a7 were grouped in cluster IV. E.a6 and E.a8 appeared to be closer to each other, with a 0.62 similarity coefficient (Tabela 3).

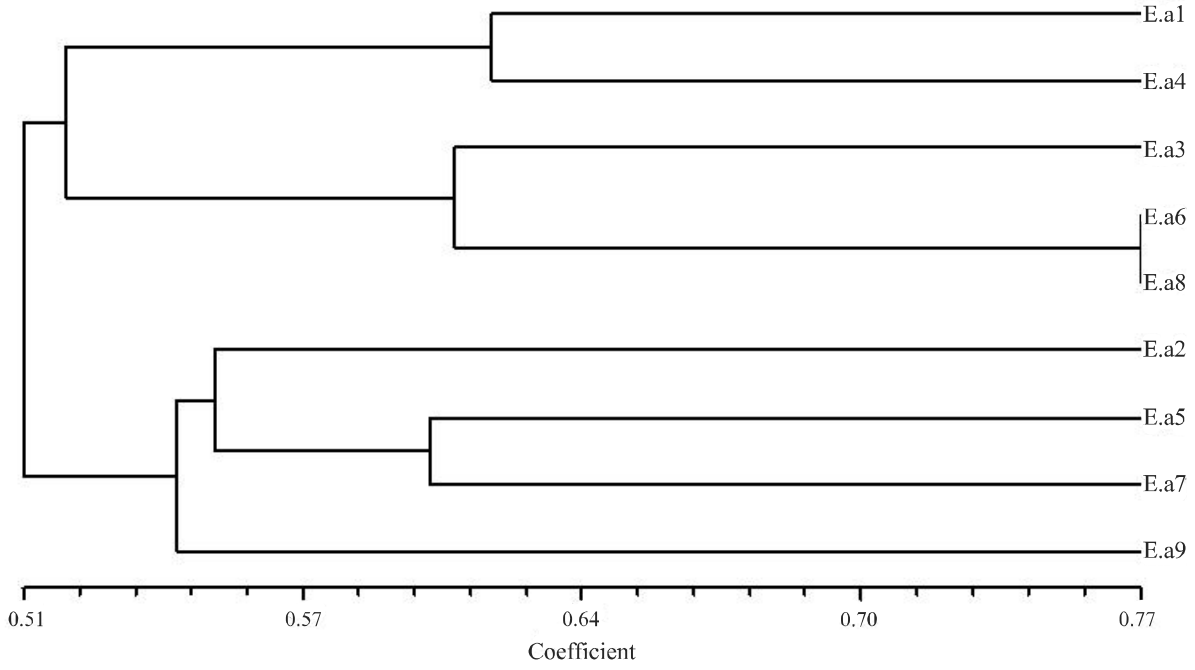
Table 2 - List of ISSR primers, the number of amplified products, the number of polymorphic and monomorphic bands, and percentage of polymorphism obtained by analyzing 9 genotypes of *E. angustifolia*

Primer	Primer sequence	Molecular weight range (bp)	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	% polymorphism
UBC811	5′-(GA)8C-3′	200 - 2500	5	3	2	60
UBC814	5-′(CT)8A-3′					
UBC818	5′-(CA)8G-3′	300 - 2500	7	5	2	71.4
UBC820	5-′(GT)8C-3′					
UBC825	5′-(AC)8T-3′	200 - 3000	15	9	6	60
UBC826	5′-(AC)8C-3′	300 - 2000	19	19	0	100
UBC827	5′-(AC)8G-3′	400 - 2500	16	14	2	87.5
UBC834	5′-(AG)8CTT-3′					
UBC836	5′-(AG)8CA-3′	500 - 2500	14	11	3	78.5
UBC840	5′-(GA)8CT-3′					
UBC841	5′-(GA)8CC-3′	600 - 2500	16	15	1	93.7
UBC845	5′-(CT)8AGG-3′					
UBC846	5-′(CA)8AGT-3′					
UBC848	5′-(CA)8AGC-3′	200 - 3000	6	6	0	100
UBC856	5′-(AC)8CTA-3′	500 - 2500	8	7	1	87.5
UBC858	5′-(TG)8AGT-3′					
UBC868	5′-CGTAGTCGT(CA)7-3′	400 - 3000	4	1	3	25
UBC888	5′-GAG(CA)7-3′	500 - 2500	6	2	4	33.3
Total			116	92	24	72.4
Mean			10.5	8.3	2.1	79

Table 3 - Jaccard's similarity coefficient among 9 *E. angustifolia* genotypes based on ISSR data analysis

	E.a1	E.a2	E.a3	E.a4	E.a5	E.a6	E.a7	E.a8	E.a9
E.a1	1.0000								
E.a2	0.5316	1.0000							
E.a3	0.5357	0.5506	1.0000						
E.a4	0.6164	0.4432	0.5517	1.0000					
E.a5	0.4778	0.5106	0.5789	0.5795	1.0000				
E.a6	0.4828	0.4526	0.5870	0.5698	0.5625	1.0000			
E.a7	0.5422	0.5930	0.4796	0.4725	0.6022	0.4646	1.0000		
E.a8	0.4884	0.4731	0.6292	0.4725	0.4900	0.7683	0.4845	1.0000	
E.a9	0.4699	0.5412	0.5109	0.6000	0.5543	0.5109	0.5333	0.4681	1.0000

Figure 3 - Dendrogram obtained with UPGMA based on Jaccard's similarity coefficient. codes represent the genotypes of *E. angustifolia* according to Table 1



ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (ZIETKIEWICZ; RAFALSKI; LABUDA, 1994). This method also may have potential for analyzing biogeographic patterns among populations of a single plant species (LI; CHEN, 2008). High reproducibility of ISSR may be due to the use of longer primers and higher annealing temperatures (MORENO; MART; ORTIZ, 1998) compared with those used normally for other DNA amplification-based techniques, such as the RAPD.

Earlier studies by Chen *et al*. (2008) using the ISSR markers for genetic diversity of 5 populations of *Hippophae rhamnoides* in China based on fingerprinting patterns revealed high levels of genetic variation within populations and subpopulations.

This is the first report of using ISSR markers in surveying genetic variation and determination of genetic relationships in the *E. angustifolia*. In the present investigation, 11 ISSR primers produced 92 polymorphic and 24 monomorphic bands (79.3% polymorphism) that unambiguously discriminated 9 *E. angustifolia* genotypes

into 5 major clusters. Results indicated the presence of wide genetic variability which reflects a high level of polymorphism at the DNA level. Variations in DNA sequences lead to polymorphism and greater polymorphism are indicative of greater genetic diversity. The ISSR method has been reported to be more reproducible (GOULAO; OLIVEIRA, 2001) and produces more marker patterns than the RAPD approach (CHOWDHURY; VANDENBERG; WARKENTIN, 2002), which is advantageous when differentiating closely related cultivars. ISSR has also been used for cultivar identification in numerous plant species, including sorghum (MEDRAOUI *et al*., 2007), apple (GOULAO; OLIVEIRA, 2001) and strawberry (ARNAU; LALLEMAND; BOURGOIN, 2003).

Based on morphological traits such as speckles on leaves, color of adaxial leaf surface, shape of leaf blade, shape of leaf apex, shape of leaf margin, shape of leave base, fruit surface type, type of fruit pedicel, presence of wings on fruit surface, shape of fruit, fruit length diameter, fruit width diameter and seed size it was predicted that there is a close relationship between Piranshahr (E.a6) and Mahabad (E.a8) genotypes which is supported by similarity coefficient of 0.76 for ISSR markers. These 2 genotypes showed a high similarity on most morphological traits and clustering using ISSR data, placed them in the same cluster, too. Band genotype (E.a9) was different from the other genotypes based on morphological traits (length of leaves, width of leaves, ripe fruit color, shape of fruit, seed size and fruit length diameter). E.a9 also formed a single cluster itself which could be due to its different genetic background. Our results show relatively high genetic distance (0.24 to 0.56) among *E. angustifolia* genotypes in West Azarbaijan province in Iran.

This is the first evaluation of genetic diversity of *E. angustifolia* in Iran. ISSR analysis was applied for the first time to obtain preliminary information on genetic diversity of this plant in the world and this is the start of further studies by more powerful markers. Earlier studies by Sheng *et al*. (2006) using the RAPD technique provided some information about the relationship between the quantity and distribution pattern of genetic variation in space and time and the geographical or ecological factors that might serve as theoretical evidences for the classification of the species or intraspecific patterns and for the evaluation of the ecological adaptability of *Hippophae L.*. In order to preserve this highly valuable plant, more samples of this plant should be collected.

CONCLUSION

In conclusion, there is a high genetic variability among studied *E. angustifolia* genotypes in West Azarbaijan province of Iran. The present study reveals that PCR based fingerprinting techniques (ISSR) are informative for estimating the extent of genetic diversity as well as determining the pattern of genetic relationships.

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