Genetic diversity among *Elaeagnus angustifolia* L. populations based on some morphological traits and Random Amplified Polymorphic DNA Markers

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Abstract
Elaeagnus angustifolia L. is a Eurasian tree that has become naturalized and has various ecological, medicinal and economical uses. In this study, a combination of morphological traits and RAPD markers were used to study the presence or absence of an association between genetic variation and morphological features among five populations of *E. angustifolia* collected from the East Azarbaijan of Iran. Data analysis of 19 different morphological traits, according to Nei's genetic distance matrix using Nei's in GenAlEx 6.5, showed that genetic distance coefficient ranged from 0.014 (between Jolfa and Ahar populations) to 0.86 (between Jolfa/Marand and Meianeh populations). The cluster analysis based on UPGMA and dendrogram plotted using NTSYSpc 2.02 software, revealed 4 main clusters. RAPD analysis using four random primers generated 29 polymorphic bands. Accordingly, the samples were placed in 4 groups. Based on Nei's genetic distance matrix, a great genetic distance existed between Jolfa and Meianeh populations (0.167) and great genetic similarity existed between Jolfa and Marand populations (0.955). In this research, the results of morphological traits and RAPD markers showed more consistent with each other. Our results showed that RAPD analysis is a suitable method to study genetic diversity and relationships among *E. angustifolia* populations.

Key words: *Elaeagnus angustifolia*, Genetic diversity, RAPD, Morphological traits, Iran.

INTRODUCTION

Elaeagnus angustifolia L. that also named Russian olive, is an Eurasian tree, native to southern Europe, central and western Asia. Within this region it occurs primarily on coasts, in riparian areas, and in other relatively moist habitats (Zouhar, 2005). *E. angustifolia* L. belongs to Elaeagnaceae family with high capacity to grow over a range of environmental conditions (Asadiar *et al.*, 2012a). The small family elaeagnaceae has three genera namely Elaeagnus L., Hippophae L. and Shepherdia Nutt and has 77 species worldwide. *E. angustifolia* is a deciduous tree, sometime shrub, erect, to 10m tall in cultivation (Sun and Lin, 2010). Various medicinal uses have been shown for *E. angustifolia*. The ripe fruits of *E. angustifolia* have been used to treat amoebic dysentery. There is general belief that leaves and fruits of the plant have antipyretic effect (Wang *et al.*, 2006). There are large variations in the content of biologically active compounds in the leaves and fruits and the tolerance to drought, salinity and alkalinity stresses among the *E. angustifolia* populations. There are a variety of molecular markers to identify the valuable *E. angustifolia* resources, and classifying the populations. Some methods such as RP-HPLC biochemical markers (Wang *et al.*, 2006), ISSR genetic markers (Assadiar *et al.*, 2012a) and RAPD molecular markers have been used to study genetic diversity and

relationship among *E. angustifolia* species (Assadiar *et al.*, 2012b). RAPD molecular markers are DNA fragments from polymerase chain reaction (PCR) amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequences. RAPD markers are able to differentiate between genetically distinct individuals (Taghizad *et al.*, 2010) and are one of the most efficient molecular methods in terms of ability to produce abundant polymorphic markers (Williams *et al.*, 1990). The advantages of RAPD are its rapidity, simplicity and do not require previous knowledge of genome (Rahman, 2006; Tucak *et al.*, 2008). So, RAPD analysis is a valuable tool in studying patterns of gene expression, inter- and intra-species genetic variations and identification of specific genes using nearly isogenic variants in plant and animal research (Caetano-Anollés *et al.*, 1991; Barker *et al.*, 1999; Kuddus *et al.*, 2002; Bauvet *et al.*, 2004; Arzani and Samei, 2004; Vandemark *et al.*, 2006). In this study, we used morphological traits and RAPD markers to investigate genetic variation among different genotypes of *E. angustifolia*.

MATERIALS AND METHODS

Plant material and DNA extraction

The leaves of *E. angustifolia* were collected from 5 different locations of East Azarbaijan province of Iran (Figure 1) in May 2012 (Table 1). Genomic DNA was extracted from dried leaves. Approximately 100 mg of samples was pulverized in a mortar and then extracted by cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The quantity and quality of each DNA bulk sample was determined spectrophotometrically at 260 nm (nanodrop 1000, Thermo Scientific) and 1% agarose gel electrophoresis.

RAPD-PCR amplification

In this study, seven RAPD primers, which were taken

Table 1. *E. angustifolia* genotypes collected from East Azarbaijan province of Iran.

Sample	Location	Latitude	Longitude	Habitat (m)
Ea1	Jolfa	42º 75'	56º 09'	965
Ea2	Jolfa	43º 04'	55º 45'	833
Ea3	Jolfa	43º11'	55º 60'	703
Ea4	Jolfa	43º 01'	57º 43'	981
Ea5	Marand	42º 53'	57º 13'	1314
Ea6	Marand	42º 46'	56º 18'	1743
Ea7	Marand	42º 72'	56º 31'	1392
Ea8	Marand	42º 43'	57º 53'	1695
Ea9	Meianeh	41º 56'	70º 75'	1486
Ea10	Meianeh	41º 64'	69º 23'	1605
Ea11	Meianeh	41º 66'	71º 25'	1696
Ea12	Meianeh	41º 63'	71º 16'	1684
Ea13	Ahar	42º 55'	66º 75'	1453
Ea14	Ahar	42º 56'	66º 77'	1445
Ea15	Ahar	42º 59'	67º 86'	1492
Ea16	Ahar	42º 64'	67º 84'	1420
Ea17	Tabriz	42º 16'	61º 75'	1512
Ea18	Tabriz	42º 22'	60º 63'	1356
Ea19	Tabriz	42º 16'	63º 08'	1525
Ea20	Tabriz	42º 18'	63º 09'	1529

Figure 1. Geographical distribution and collection sites of *E. angustifolia* genotypes in East Azerbayejan province of Iran.

Table 2. Primer sequences and RAPD products generated by primers in *E. angustifolia* genotypes.

Primer	Primer sequence	GC (%)	TM	Number of amplified bonds	Number of polymorphic bonds	Polymorphic/amplified bondnds (%)
RP_1	5′TGCCCGTCGT 3′	70	34	10	10	100
RP_2	5′ACAACGCCTC 3′	60	32	--	--	--
RP_3	5′TGCCGAGCTG 3′	70	34	8	8	100
RP_4	5′GGGTAACGCC 3′	70	34	9	8	88.88
RP_5	5′GGTGAACGCT 3′	60	32	--	--	--
RP_6	5′GGACCCAACC 3′	70	34	5	5	100
RP_7	5′TGCGCCCTTC 3′	70	34	--	--	--

Table 3. Morphological characters and their code for preparing matrix data.

No.	Traits
1	Length of leaves: ≤ 6 cm (0); 6 to 7 cm (1); > 7cm (2)
2	Width of leaves: ≤ 1.3 cm (0); 1.3 to1.7 cm (1); > 1.7cm (2)
3	Length of leaves pedicel: ≤ 9 mm (0); 9 to 11 mm (1); > 11 (2)
4	Speckles on leaves: absent (0); present (1)
5	Color of abaxial leaf surface: yellowish white (0); silvery (1); rust- colored or ferruginous (2)
6	Color of adaxial leaf surface: yellowish white or silvery (0); rust- colored or ferruginous (1)
7	Shape of leaf blade: round or ovate (0); oblong or elliptic (1); lanceolate (2)
8	Shape of leaf apex: round or obtuse (0); acute or acuminate (1)
9	Shape of leaf margin: revolute (0); entire (1)
10	Ripe fruit color: red (0); yellowish brown or yellow to orange (1); yellowish gray (2)
11	Fruit surface type: hairy (0); scaly (1)
12	Type of fruit pedicel: erect, robust (0); slender, nodding (1)
13	Presence of wings on fruit surface: absent (0); present (1)
14	Shape of fruit: nearly globose or obovoid (0); ellipsoid (1); ovate (2)
15	Length of fruit pedicel: ≤ 2mm (0); 2 to 6mm (1); > 6 mm (2)
16	Fruit length diameter: ≤ 4.6cm (0); 4.6 to 6cm (1); > 6 (2)
17	Fruit width diameter: ≤ 3.9cm (0); 3.9 to 4.4 (1); > 4.4cm (2)
18	Shape of seed: narrow and long (0); ovate (1)
19	Seed size: ≤ 1.5cm (0); 1.5 to 1.8cm (1); > 1.8 cm (2)

from various previous studies, were used (Table 2). The reaction mixture for RAPD amplification assay had a total volume of 20 µl, which contained 40 ng genomic DNA, 10 µl Master mix (from BIORON company, containing 1 unit *Taq DNA polymerase*, 0.1 mM of each dNTPs, 2.5 mM $MgCl_2$, 0.01% Tween 20, 65 mM Tris-HCl and 16 mM $(NH_4)_2SO_4$ and 70 pmol primer. The amplification was carried out on a gradient thermo cycler (LabCycler/SensoQuest, Germany), with an initial step of 5 min denaturation at 94°C, followed by 45 cycles of 45 s at 94°C, 1 min at 37°C and 90 s at 72°C, and a final extension step for 7 min at 72°C. The PCR amplified products were run on a 2% agarose gel containing safe dye stain in 1× TBE buffer for 2h. Then gels were digitally photographed under ultraviolet light in a transilluminator documentation system (Gerix 1000, Biostep).

RAPD data analysis

DNA banding patterns generated were scored for the presence (1) or absence (0) of each amplified band to create binary data matrices. To assess the genetic relationships between populations based on Nei's genetic distance coefficients (Nei, 1973), NTSYS- pc 2.02 software was used to construct UPGMA (Unweighted Pair Group Method of Cluster Analysis) – dendrogram.

Morphological traits

Nineteen morphological characteristics were considered to evaluate genetic diversity as described before by

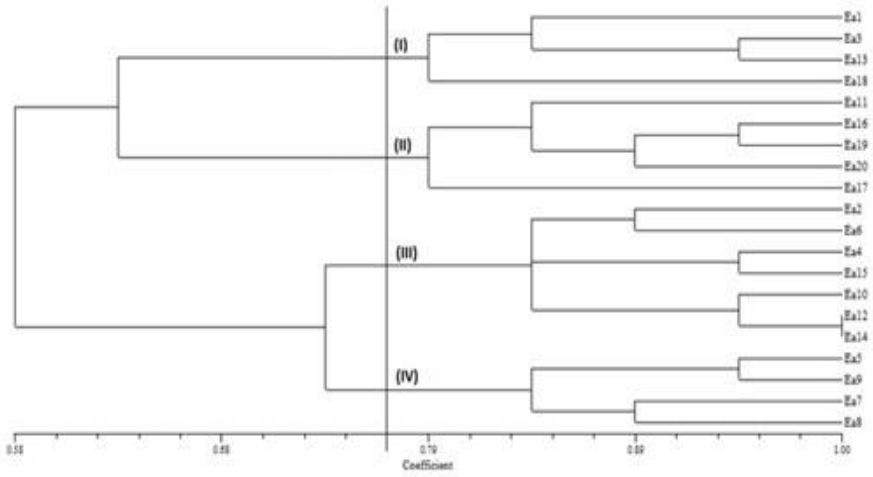

Figure 2. Dendrogram generated using UPGMA method showing relationships between populations of *E. angustifolia* using morphological characters data.

Table 4. Nineteen morphological characters used for construction of cluster analysis for *E. angustifolia* genotypes (C: character).

Sample	C: 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9
E.a1	0 1 0 1 1 0 0 0 1 1 0 0 0 0 1 1 0 1 0
E.a2	0 1 0 1 1 0 1 1 1 0 0 0 0 0 1 1 0 1 1
E.a3	0 1 0 1 1 0 0 0 1 1 0 0 0 0 1 1 0 0 0
E.a4	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 1 0
E.a5	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 0 0
E.a6	0 1 0 1 1 0 1 1 1 0 0 0 0 1 1 1 0 1 0
E.a7	0 1 0 1 1 0 1 1 1 1 0 0 0 1 1 1 0 0 0
E.a8	0 1 0 1 1 0 1 1 1 0 0 0 0 1 1 1 0 0 0
E.a9	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 1 0
E.a10	0 1 0 1 1 0 1 1 1 0 0 0 0 0 1 1 0 0 0
E.a11	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 0 1
E.a12	0 1 0 1 1 0 1 1 1 0 0 0 0 0 1 1 0 0 0
E.a13	0 1 0 1 1 0 0 0 1 1 0 0 0 0 1 1 0 0 0
E.a14	0 1 0 1 1 0 1 1 1 0 0 0 0 0 1 1 0 0 0
E.a15	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 1 0
E.a16	0 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 0 0
E.a17	1 1 0 1 1 0 1 1 1 1 1 1 0 0 1 1 0 0 0
E.a18	0 1 0 1 1 0 1 0 1 1 0 1 0 0 1 1 0 0 0
E.a19	0 1 0 1 1 0 1 1 1 1 0 1 0 0 1 1 0 0 0
E.a20	1 1 0 1 1 0 1 1 1 1 0 0 0 0 1 1 0 0 0

Asadiar *et al.* (2012) (Table 3). Some traits that were identical among genotypes were not considered for further analysis (Tables 4 and 5).

RESULTS AND DISCUSSION

Matrix data was prepared according to the morphological states (Table 4). Cluster analysis of 19 morphological traits showed that the genotypes of *E. angustifolia* divided into 4 main clusters (Figure 2). Each of I, II and IV clusters was divided into two sub clusters while cluster III was subdivided into three sub clusters. In cluster III, E.a12 and E.a14 were common in all morphological traits. According to Nei's genetic distance matrix using Nei in GenAlEx 6.5, genetic distance coefficient ranged from 0.014 (between Jolfa and Ahar populations) to 0.086 (between Jolfa/Marand and Meianeh populations). Out of seven RAPD primers used in this study, four primers produced reproducible bands. A total of 29 polymorphic bands were identified ranging from 100 to 1500 bp. Three primers (RP1, RP3 and RP6) produced 100% polymorphic bands (Table 2). The Cluster analysis, using RAPD data showed that the genotypes of *E. angustifolia* were divided into 4 main clusters (Figure 3).

According to the previous plant classification based on morphological traits, each plant depends on morphological properties based on one classification unit. In this study, combination of morphological and genetically parameters were used for analyzing the relationship between the populations. Maximum calculated genetic distance based on Nei genetic distance matrix using Gen Alex 6.5 software was 0.086 between Jolfa and Meianeh populations; and maximum genetic similarity matrix was 0.986 between Ahar and Jolfa populations, according to morphological data (Tables 5 and 6). Data analysis using NTsyspc 2.02 showed that genetic distance dendrogram was including four major groups. Also, according to RAPD data analysis, Maximum calculated genetic distance was

Table 5. Similarity matrix for *E. angustifolia* populations based on morphological characters.

	E.a1	E.a2	E.a3	E.a4	E.a5	E.a6	E.a7	E.a8	E.a9	E.a10	E.a11	E.a12	E.a13	E.a14	E.a15	E.a16	E.a17	E.a18	E.a19	E.a20
E.a1	1.000																			
E.a2	0.736	1.000																		
E.a3	0.842	0.684	1.000																	
E.a4	0.842	0.894	0.789	1.000																
E.a5	0.786	0.736	0.842	0.842	1.000															
E.a6	0.736	0.894	0.684	0.894	0.736	1.000														
E.a7	0.684	0.736	0.842	0.842	0.894	0.842	1.000													
E.a8	0.684	0.736	0.736	0.736	0.894	0.842	0.894	1.000												
E.a9	0.842	0.789	0.789	0.894	0.947	0.789	0.842	0.842	1.000											
E.a10	0.684	0.842	0.842	0.842	0.894	0.842	0.894	0.894	0.842	1.000										
E.a11	0.736	0.894	0.789	0.894	0.842	0.789	0.842	0.736	0.789	0.842	1.000									
E.a12	0.736	0.894	0.789	0.894	0.842	0.894	0.842	0.842	0.789	0.947	0.894	1.000								
E.a13	0.894	0.736	0.947	0.842	0.789	0.736	0.789	0.684	0.736	0.789	0.842	0.842	1.000							
E.a14	0.736	0.894	0.789	0.894	0.842	0.894	0.842	0.842	0.789	0.947	0.894	1.000	0.842	1.000						
E.a15	0.789	0.842	0.842	0.947	0.894	0.842	0.894	0.789	0.947	0.894	0.842	0.842	0.789	0.842	1.000					
E.a16	0.842	0.789	0.789	0.894	0.947	0.789	0.842	0.842	0.894	0.842	0.894	0.894	0.842	0.894	0.842	1.000				
E.a17	0.631	0.684	0.684	0.789	0.736	0.684	0.736	0.631	0.684	0.736	0.789	0.789	0.736	0.789	0.736	0.789	1.000			
E.a18	0.789	0.578	0.842	0.684	0.842	0.578	0.736	0.736	0.789	0.736	0.684	0.684	0.789	0.684	0.736	0.789	0.684	1.000		
E.a19	0.789	0.736	0.736	0.842	0.894	0.736	0.789	0.789	0.842	0.789	0.842	0.842	0.789	0.842	0.789	0.947	0.842	0.842	1.000	
E.a20	0.789	0.736	0.736	0.842	0.894	0.736	0.789	0.789	0.842	0.789	0.842	0.842	0.789	0.842	0.789	0.947	0.842	0.736	0.894	1.000

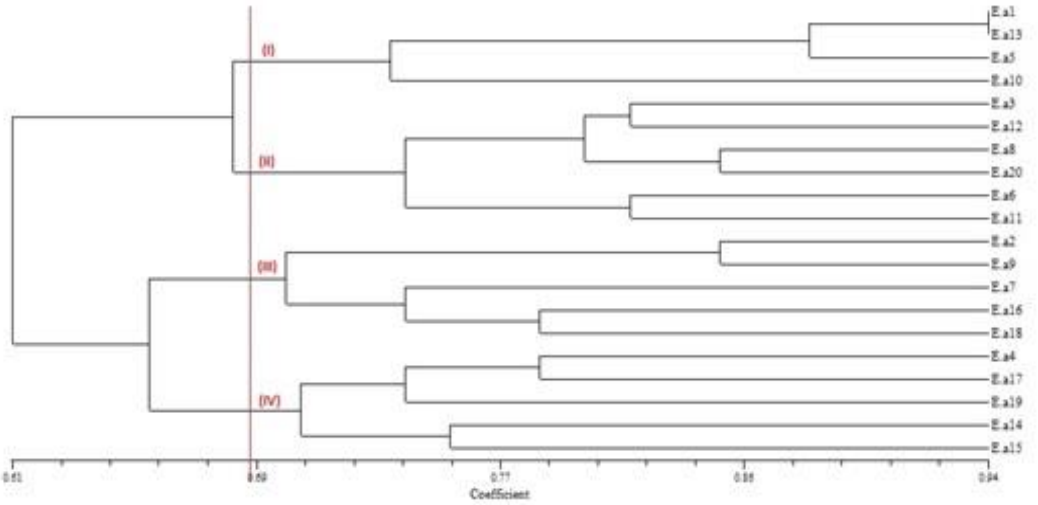

Figure 3. Dendrogram generated using UPGMA method showing relationships between populations of *E. angustifolia* using RAPD marker data.

Table 6. Pairwise population matrix of Nei's Genetic identity based on morphological characters.

Population	Jolfa	Marand	Tabriz	Ahar	Mianeh
Jolfa	0.000				
Marand	0.085	0.000			
Tabriz	0.069	0.015	0.000		
Ahar	0.014	0.046	0.032	0.000	
Meianeh	0.086	0.081	0.066	0.052	0.000

Table 8. Pairwise population matrix of Nei's Genetic distance based RAPD data.

Population	Jolfa	Marand	Tabriz	Ahar	Meianeh
Jolfa	0.000				
Marand	0.046	0.000			
Tabriz	0.075	0.094	0.000		
Ahar	0.068	0.052	0.111	0.000	
Mianeh	0.167	0.146	0.150	0.152	0.000

0.167 between Jolfa and Meianeh populations; and maximum genetic similarity matrix was 0.955 between Ahar and Jolfa populations (Tables 7 and 8). In this study, the morphological data and RAPD data are consistent with each other. Existing high similarity between *E. angustifolia* populations could be due to ecological conditions in regions.

Since *E. angustifolia* is tolerant to severe drought, high salinity and alkalinity in soils, it is said to play a very important role in maintaining ecosystem function in the hyper arid areas. It is also used for various medicinal purposes (Zhang and Zhao, 1996). Therfore, various types of biochemical, morphological and molecular markers have been widely used for the analysis of genetic diversity among and between *E. angustifolia* populations worldwide. Wang *et al.* (2006) used RP-HPLC (reversed-phase high-performance liquid chromatography) for the classification and analysis of intra-specific genetic relationships of seventeen populations of *E. angustifolia*. Asadiar *et al.* (2012b) evaluated genetic relationships and polymorphism among genotypes of *E. angustifolia* collected from different locations of West Azarbaijan province using a combination of morphological traits and molecular RAPD marker. Uzun *et al.* (2015) used a combination of fruit characteristics, RAPD and ISSR markers for the evaluation of genetic variation among 56 *E. angustifolia* accessions collected from the Central Anatolian region.

Today, genetic markers are widely used for genetic diversity studies. In addition, comparison between the molecular markers is inevitable.

The present study showed that RAPD markers provide some useful information about relationship between *E. angustifolia* populations, the distribution

Table 7. Similarity matrix for *E. angustifolia* populations based on RAPD data.

	E.a1	E.a2	E.a3	E.a4	E.a5	E.a6	E.a7	E.a8	E.a9	E.a10	E.a11	E.a12	E.a13	E.a14	E.a15	E.a16	E.a17	E.a18	E.a19	E.a20
E.a1	1.000																			
E.a2	0.515	1.000																		
E.a3	0.666	0.606	1.000																	
E.a4	0.363	0.727	0.636	1.000																
E.a5	0.848	0.606	0.818	0.515	1.000															
E.a6	0.636	0.757	0.666	0.666	0.666	1.000														
E.a7	0.545	0.666	0.575	0.757	0.515	0.787	1.000													
E.a8	0.666	0.606	0.818	0.575	0.696	0.727	0.696	1.000												
E.a9	0.606	0.848	0.696	0.575	0.696	0.666	0.636	0.757	1.000											
E.a10	0.787	0.606	0.696	0.454	0.696	0.727	0.575	0.636	0.696	1.000										
E.a11	0.515	0.636	0.787	0.727	0.606	0.818	0.666	0.787	0.606	0.606	1.000									
E.a12	0.666	0.666	0.818	0.636	0.757	0.787	0.636	0.757	0.696	0.757	0.787	1.000								
E.a13	0.939	0.575	0.727	0.424	0.909	0.696	0.606	0.727	0.666	0.727	0.575	0.727	1.000							
E.a14	0.303	0.666	0.575	0.696	0.454	0.606	0.575	0.575	0.636	0.515	0.666	0.575	0.363	1.000						
E.a15	0.363	0.545	0.636	0.696	0.515	0.606	0.575	0.575	0.575	0.575	0.666	0.575	0.424	0.757	1.000					
E.a16	0.575	0.757	0.666	0.606	0.606	0.696	0.727	0.606	0.727	0.606	0.636	0.666	0.636	0.666	0.606	1.000				
E.a17	0.515	0.636	0.727	0.787	0.606	0.757	0.727	0.666	0.606	0.606	0.818	0.787	0.575	0.666	0.787	0.696	1.000			
E.a18	0.484	0.666	0.696	0.696	0.575	0.606	0.757	0.757	0.757	0.515	0.727	0.696	0.545	0.696	0.696	0.787	0.787	1.000		
E.a19	0.454	0.636	0.545	0.727	0.484	0.696	0.727	0.666	0.606	0.545	0.636	0.606	0.515	0.727	0.666	0.636	0.757	0.727	1.000	
E.a20	0.696	0.696	0.787	0.666	0.727	0.696	0.666	0.848	0.727	0.666	0.696	0.848	0.757	0.484	0.545	0.636	0.757	0.727	0.696	1.000

pattern, genetic variation and the geographical and ecological factors. They might also provide data for the taxonomy of the species or intraspecific relationship patterns and for the evaluation of the ecological adaptation of *E. angustifolia.*

In conclusion, molecular variation assessed in this study in combination with morphological characters of *E. angustifolia* can be useful in conventional and molecular breeding programs for this medicinal plant. But it is suggested that: (1) More samples of this plant should be collected for further genetic variation studies; (2) In addition to other molecular markers, pollen micromorphological studeis can also be used for genetic diversity assessments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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