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Original article

Essential oil diversity and molecular characterization of *Ephedra* species using RAPD analysis

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Abstract

Background and objectives: The genus *Ephedra* (Ephedraceae) consists of about 40 species of mostly shrubs and rarely small trees around the world. In the present study, the essential oil (EO) diversity and genetic relationships were investigated in six *Ephedra* species from Iran using Random Amplified Polymorphic DNA (RAPD) markers. **Methods:** The plants were collected from two different provinces; Azarbayjan (north-west) and Khorasan (north-east) of Iran. The EOs were obtained by hydro-distillation and analyzed by GC and GC/MS. The DNA was extracted from the aerial parts of the plants using a Qiagen Dneasy Plant Mini Kit. Amplification was performed using decamer RAPD primers**. Results:** A total of 187 bands were scored and used for the analysis of genetic distances. Genetic distance values ranged from 0.25 to 0.95.The analysis showed the highest genetic diversity (25%) between *E. foliata* with other species. *Ephedra foliata* formed a distinct group. *Ephedra strobilacea* was found to be the most similar to *E. sarcocarpa* (male).**Conclusion**: High genetic and EO diversity was demonstrated in this genus which should be further studied in order to make more efficient use of the species and considering relevant conservation programs.

Keywords: DNA amplification, *Ephedra*, essential oil, genetic distance, RAPD markers

Introduction

Ephedra L. is the only genus in its family Ephedraceae. The genus has about 40 species distributed in arid regions of Eurasica and the America [1]. These plants are xeromorphic equistoid shrubs, climbers or small trees. The leaves are opposite or in whorls of 3[2] and are scale-like and evanescent. Female inflorescence sometimes become swollen and juicy and consists of 1-3 flowers. Male flower are subtended by a bract, microsporagiophor, forked or even three. The cones are solitary or paired forming syncorp with two pairs of yellow or red bracts membranous [1,3]. Eight species of this genus grow in Iran one of which, *E. laristanica*

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Assadi, is endemic to the country [2,4]. *Ephedra* species are mainly distributed in different parts of the country, belonging to the Irano- Turanian phytogeographical region [4]. In Iran the key character separating thesespecies is the morphological characterization. *Ephedra foliata* Boiss. & Kotschy ex Boiss. is a shrub with trailing or scandent stem 3-5 m long. The branches are subverticillate or fascicled, thin, persistent, with long enternodes. It grows in Balochistan and Fars Provinces, altitude 1500 m [4,5]. *Ephedra intermedia* Schrenk & C.A.Mey. is a shrub, up to 1 m tall, branches densely verticillate, growing in Balochistan and Khorasan provinces, altitude 1500 m [4,6]. *Ephedra distachya* L. is a subshrub with creeping rootstock, growing in the north (Manjil), east-north (Damghan), east (Harirud valley) and east-central parts (Yazd and Lut desert) of Iran, altitude 100 m [4,7]. It is remarkable that *E. intermedia* and *E. distachya* show many similarities, although they grow in distinct regions. *Ephedra strobilacea* Bunge is an erect dioecious, much-branched shrub, 1-2 m tall, growing in Balochistan, Fars, Kerman, Khouzestan and Bushehr provinces, Iran, altitude 800-2100 m [4,6]. *Ephedra sarcocarpa* Aitch. & Hemsl. is a shrub, up to 1 m high, growing in east (Harirud valley), east-north (Semnan) and south (Firuzabad in *Fars* province) of the country, altitude 100-1500 m [4,7]. *E. major* Host is a dioecious shrub with an erect or occasionally prostrate stem 20-150 cm tall, growing in different parts of Iran, altitude 1500-3000 m [4,5]. It is notable that *E. major*iswidely is distributed in the world with significant variations in morphological characteristics which are sometimes correlated with the geographic region and habitat conditions [4]. In traditional Chinese medicine, the genus *Ephedra* has been used for 5000 years to treat allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion [8]. Recently, much attention has been paid to the *Ephedra* species and their chemical constituents because of diverse activities such as antiviral [9], hepatoprotective [10], gastric ulcer healing [11], antifungal [12], hypoglycemic [13], antimicrobial and antioxidant [14] properties. Genetic and EO diversity investigations of the *Ephedra* species are useful and important for the efficient use and conservation of these resources. RAPD is one of the PCR based techniques that have been used successfully in DNA fingerprinting of plant genomes and in genetic diversity studies [15]. Examples are investigating genetic features of seven *Ephedra* species, growing in the south of Kazakhstan [16] or using RAPD markers for assessing genetic diversity in *Ephedra* species growing in Pakistan [17]. To the authors best knowledge molecular data available on *Ephedra* species from Iran is very limited so far. Therefore, the present work was carried out to investigate the EO diversity and genetic relationships of six *Ephedra* species from Iran using RAPD markers.

Experimental

Plant material

Plant materials were obtained from Razavi Khorasan (Dehbar village, Bazangan lake and Arefy village), South Khorasan (Ghaen, between Ariyanshahr and Birjand) and Eastern Azarbayjan (Moghan) provinces, Iran and identified by Mrs. A. Ansari, from the Herbarium of the Faculty of Pharmacy, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran, where voucher specimens have been deposited (table 1). Fresh aerial parts of both male and female of each species were preserved at -80 °C until DNA extraction.

Isolation of the essential oil

The EOs from dry powdered aerial parts (leaves, stems and flowers) of both male and female of each species (100 g) were isolated by hydro-distillation for 3 h, using a Clevenger-type apparatus. The distillated oil was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at -20 °C until analysis by gas chromatography-mass spectrometry (GC/MS).

Table 1. List of location, altitude, collection date and voucher number of *Ephedra* samples studied

Species	Persian name	Location and altitude (m)	Collection date	Voucher No.
E. major Host	*Rish boz*	Dehbar village, Razavi Khorasan province, 1783 m.	May 2011	12820
E. strobilacea Bunge	*Ormak biyabani*	Zirkooh, Ghaen, South Khorasan province, 888 m.	May 2011	12821
E. sarcocarpa Aitch. & Hemsel.	*Ormak noghreyi*	Between Ariyanshahr and Birjand, South Khorasan province, 1711 m.	June 2011	12822
E. foliate Boiss. & Kotschy ex Boiss.	*Ormak ravandeh*	Bazangan lake, Razavi Khorasan province, 800 m.	May 2011	12823
E. intermedia Schrenk & C.A.Mey.	*Ormak miyaneh*	Arefy village, Razavi Khorasan province, 1266 m.	May 2011	12824
E. distachya L.	*Ormak doradify*	Moghan, AzerbayejanProvince, 600 m.	May 2010	12825

GC/MS analysis

The EOs were analyzed using a Varian CP-3800 gas chromatograph (USA) equipped with two flame ionization detectors (FIDs) and fused silica column: CP-Sil 8 CB (50 m × 0.25 mm, 0.12 µm film thickness). Operating conditions were as follows: oven temperature 50 °C (5 min), 3 °C/min to 250 °C (10 min); flow rate of 2 mL/min (Helium); injector temperature 260 °C; detector temperature 280 °C; 0.1 µL injection volume at split ratio 1:50. The gas chromatograph was coupled to an Agilent 5975 (Agilent Technologies, Palo Alto, CA, USA) mass selective detector equipped with a non-polar Agilent HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 250 °C and mass range from 35-465 u. Retention indices were calculated for all components using a homologous series of *n*-alkanes (C_8-C_{20}) injected in conditions equal to samples.

Identification of oil components was made by comparing their mass spectra with those from NIST, Wiley and Adams mass spectra libraries, by AMDIS (Automated Mass Spectral Deconvolution and Identification System) and by comparing their retention times with those of authentic standards in the literature [18]. The percentage ratio of EOs components was computed by the normalization method of the GC/FID peak areas and average values were taken into further consideration (n = 3).

DNA extraction

Total genomic DNA was extracted from the leaves using the Dneasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer instruction. Quantification of genomic DNA was performed by a spectrophotometer (Nanodrop ND-1000, USA), at an absorbance level of 260 nm. The quality of DNA was determined by electrophoresis on agarose gel (0.8%).

PCR amplification with RAPD primers

In this study, 9 random decamer primers (Symbion, Denmark) were taken initially and utilized for the amplification from all studied species. Sequence information for the primers has been presented in table 2. Polymerase Chain Reactions (PCR) were carried out in 25 µL reaction volume containing 1.5 mM $MgCl_2$, 1X PCR buffer, 0.2 µM of each primer, 0.2 mM dNTPs, 0.5- 2 µL (50 ng) DNA template, 1 U Taq DNA polymerase and ultrapure distilled water to a final volume of 25 µL. A control PCR tube containing all components, but no genomic DNA, was tested with each primer to check for contamination. The amplification was conducted in a thermal cycler (Techne Research Inc., UK) and programmed for an initial denaturation at 94 °C for 1.5 min and 40 cycles as follows: 2 min at 38 °C, 2 min at 72 °C and 1 min at 91 °C. Two additional steps were used: 38°C (2 min) and 72 °C (5 min) for final extension. Amplification products were separated with electrophoresis on 1.5% agarose gel (Sigma, Germany) in 1X TAE

buffer and visualized under ultraviolet light after staining with ethidium bromide.

Table 2. Detailed information of randomly amplified polymorphic DNA (RAPD) primers used in this study

NO.	Primer name	Sequences (5'-3')	Size (nt)	MW (g/mol)	Tm (°C)	GC%
1	GLA02	TGCCGAGCTG	10	3044	33.0	70
2	GLA03	AGTCAGCCAC	10	2997	28.9	60
3	GLA05	AGGGGTCTTG	10	3099	28.9	60
4	GLA08	GTGACGTAGG	10	3108	28.9	60
5	GLB10	CTGCTGGGAC	10	3044	33.0	70
6	GLB19	ACCCCCGAAG	10	2982	33.0	70
7	GLB20	GGACCCTTAC	10	2988	28.9	60
8	GLC09	GTCCCGACGA	10	3013	33.0	70
9	GLD07	TTGGCACGGG	10	3084	33.0	70

Notes: nt=number of nucleotides in a primer; MW=molecular weight in gram per mol; Tm=melting temperature; GC% = percentage of guanine and cytosine nucleotides

Data analysis

Consistency of PCR-amplified products was checked by performing all reactions three times. Bands that were unambiguous and reproducible in successive amplifications were selected for scoring. Bands were scored as "1" for presence and "0" for absence. From the binary data, a matrix of genetic similarities or distances was generated according to Nei & Li [15] and a cluster analysis was performed to generate a dendrogram according to UPGMA (unweighted pair group method of arithmetic means) method

Results and Discussion

The hydro-distillation method was used to extract the EOs of aerial parts (leaves, stems and flowers) of both male and female of five different *Ephedra* species of Iran. The obtained oils were analyzed using GC and GC/MS. The major constituents identified in each oil have been listed in table 3. It was not possible to extract any EO from the aerial parts of the *E. strobilacea* species. Nine RAPD primers were used for the genetic characterization of the selections and yielded 439 fragments with an average of 10.3 fragments per primer (figure 1).

Genetic similarities among six *Ephedra* species using RAPD primers have been presented in table 4. Genetic distance values between six *Ephedra*

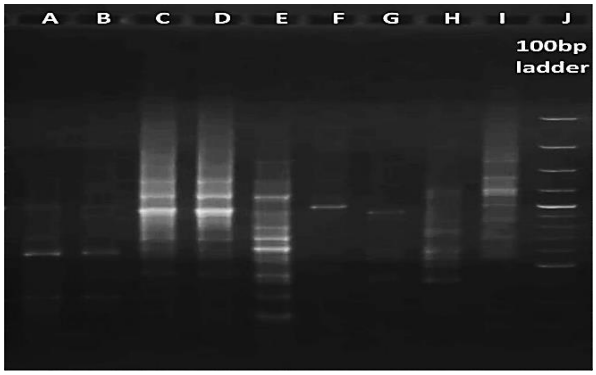

Figure 1. DNA amplification products obtained using primer GLA02. *Lane A=E. foliata* (male); *Lane B=E. foliata* (female); *Lane C=E. intermedia* (male); *Lane D=E. intermedia* (female); *Lane E=E. major* (male); *Lane F=E. major* (female); *Lane G=E. sarcocarpa* (male); *Lane H=E. sarcocarpa* (female); *Lane I=E. distachya* (male); *Lane J* =100-bp ladder molecular weight standard

species ranged from 0.25 (*E. strobilacea* and *E.sarcocarpa*) to 0.95 (*E. foliata* and *E. strobilacea*). According to Nei-Li's coefficient, the genetic similarity value was 70% for *E. strobilacea* and *E. sarcocarpa* (male), and 90% for *E. sarcocarpa* (female) and *E. strobilace* species. The similarity coefficient between *E. intermedia* and *E. major* was 84% while it was 80% between *E. intermedia* and other species.

A dendrogram based on corresponding genetic distance among the tested *Ephedra* species (figure 2) showed two major groups that segregated at a genetic distance level of 0.95. The first group (I) included *E. Foliata*. The second large group (II) was further partitioned into two subgroups (IIa and IIb). Subgroup IIa consisted of *E. intermedia* and *E. major*, whereas subgroup IIb contained *E. sarcocarpa* and *E. strobilacea*.

The major constituents (compounds representing more than 5% of the total compounds identified in the EOs of each plant) of the oils have been listed in table 1 in order of elution from the DB5 column. In our study, the EOs of investigated samples were of the monoterpenic type and significant variations in the amount of the main compounds were observed. The major monoterpenes identified in the oils obtained from aerial parts of these plants were carvone, limonene and citronellol, respectively.

Table 3. Chemical composition of the essential oils obtained by hydro-distillation from *Ephedra* species of Iran

No.	Components	KI_{ca}	KI_{ad}	Percentage				
				E. major	*E. sarcocarpa*	*E. foliata*	*E. intermedia*	*E. distachya*
1.	Camphene	956	954	-	-	nm	14.6	-
2.	ρ -Cymene	1024	1024	-	-	9.5	11.2	nm
3.	Limonene	1028	1029	nm	nm	28.5	35.7	nm
4.	1,8-Cineol	1033	1031	-		8.0	9.6	-
5.	Camphor	1149	1146	nm	nm	11.2	5.9	nm
6.	α-Terpineol	1188	1188	nm	-	16.0	8.8	17.3
7.	Myrtenol	1195	1195	nm	-	8.6	7.0	14.6
8.	Citronellol	1229	1225	21.4	-	-	-	-
9.	Carvone	1241	1243	-	53.3	-	-	-
10.	Piperitone	1252	1252	nm	13.5	-	-	nm
11.	Thymol	1288	1290	-	6.5			-
12.	Carvacrol	1297	1299	nm	16.3	-	-	nm
13.	(3Z)-3-Hexenyl benzoate	1566	1566	17.6	-	-	-	-
14.	Hexadecanoic acid	1965	1960	nm	-	-	-	14.4

KI_{ca} = Calculated kovats index: retention indices relative to homologous series of $C_8 - C_{20}$ *n*-alkanes on HP-5MS capillary column; KI_{ad}= Relative kovats index taken from Adams; (-) = not found; nm= not major (<5%).

Our results revealed significant differences between all samples in main constituents of their EOs except for *E. foliate* and *E. intermedia*. Citronellol (21.4%) and (3Z)-3-hexenyl benzoate (17.6%) were found to be the main components of the EO of *E. major*.

Carvone (53.3%) and carvacrol (16.3%) were the main constituents of the EO of *E. sarcocarpa*. Similar composition in *E. foliate* and *E. intermedia*EOs, were observed; both of them were rich in limonene (25.5% and 35.7 0%, respectively). In addition, some other compounds such as ρ-cymene, 1,8-cineol, camphor, α-terpineol and myrcenol were found in high amounts in their EOs; while α-terpineol (17.3%) and myrcenol (14.6%) were found as the major constituents of the EO of*E. distachya.*Similar to our results, the components identified in the EOs obtained from different species of *Ephedra*were different from published data.

Ji *et al*. analyzed the EOs from the dried stems of *Ephedra sinica, E. intermedia* and *E. equisetina* using GC and GC/MS [19]. The major constituents of the oils were l-alpha-terpineol in *E. sinica*, 1, 4-cineole in *E. intermedia* and hexadecanoic acid in *E. equisetina*. The EO composition of three Italian species of *Ephedra* was investigated by Kobaisya *et al*. [20]. The main constituent reported in the oil of E. *distachya* was ethyl benzoate. (E)-phytol in the oil of *E. fragilis* and eugenol in the oil of *E. major* were the major constituents.

Table 4. Genetic distance values belong to six *Ephedra* species from Iran (1: *E. distachya*; 2: *E. major*; 3: *E. intermedia*; 4: *E. foliate*; 5: *E. sarcocarpa*; 6: *E. strobilacea*)

Species	1	2	3	4	5	6
1	*					
2	0.60	*				
3	0.60	0.35	*			
4	0.90	0.85	0.88	*		
5	0.55	0.70	0.70	0.90	*	
6	0.50	0.65	0.65	0.95	0.25	*

In another study in central Italy, Maggi *et al*. analyzed the composition of the EO of*E. nebrodensis* Tineo ex Guss. subsp. *nebrodensis* [3]. They found citronellol and ethyl hexadecanoate as the main representatives in this fraction. These differences are common, since studies have shown that the oil composition of individual plants growing in different parts of the world may vary widely due to the climate, growing area, time of collection, inter-species biodiversity, seasonal and geographic conditions, etc [21].

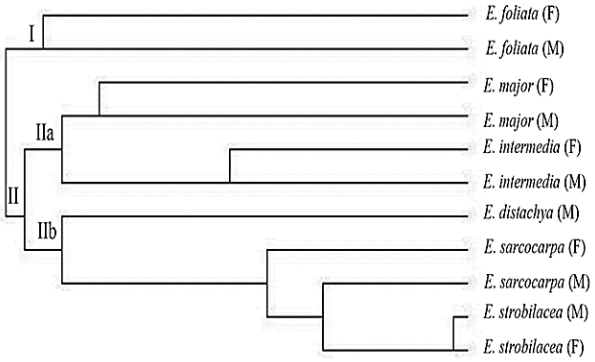

Figure 2. UPGMA dendrogram generated based on the cluster analysis of 187 RAPD bands of six species of *Ephedra*

In this study, genetic variations of six *Ephedra* species from Iran were investigated using RAPD method. *Ephedra foliata* was found to possess considerable genetic differences from other species (group I in dendrogram) that is probably due to diversity of habitat condition of this species in the Bazangan Lake. The subgroupIIa, including *E. intermedia* and *E. major*, showed 84% similarity value, although there was no similarity in their EO compositions. However, this genetic similarity is in accordance with their minute morphological character differences. While *E. intermedia* and *E. distachya* that have shown many morphological character similarities, were found to possess considerable DNA differences. They were grouped in subgroup IIa and IIb with 80% genetic similarity value. *E. strobilacea* and *E. sarcocarpa* (male) were the most genetically related species with 98% similarity value. However, in general, it seems genetic had no influences on the chemical variability of the EOs.

Based on our study, high diversity of genetic and EO content was demonstrated among all investigated samples which should be studied further in order to be able to make more efficient use of this plant resource and for the relevant conservation programs.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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