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Original Article

Assessment of Genetic Diversity by Application of Inter Simple Sequence Repeat (ISSR) Primers on Iranian Harmal (*Peganum harmala* L.) Germplasm as an Important Medicinal Plant

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Abstract

Syrian Rue or Harmal (*Peganum harmala* L.), belonged to the family of Peganaceae, grows in semi-arid climates such as the Middle East and North Africa. Traditionally, this plant, especially the seeds, has been recognized for its several medicinal uses. In this stydy, genetic diversity between 21 Harmal accessions, collected from different regions of Iran were evaluated by Inter Simple Sequence Repeat (ISSR) marker using 14 spesific primers. All primers successfully amplified polymorphism regions, as among 115 regions, 68 polymorphic regions (59.13%) were amplified. Accordingly, highest and lowest similarities among ecotypes were measured as 94% and 55%, respectively. The results based on cluster analysis also categorized all accessions into three groups that did not completely match to their geographic pattern place of collection. Result of principle coordinate analysis of samples also showed distribution pattern similar to cluster analysis.

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Introduction

Harmal (*Peganum harmala* L.) grows in semi-arid climates such as the Middle East and North Africa [1]. As fully described in several studies, this perennial plant has many medicinal properties including: insecticide features of seed extract [2], use in relieve dolorous process [3], antibacterial properties [4], and inhibition of osteoclast differentiation [5]. Conserving genetic reservoir is a necessary requisite for all plant breeding strategies in order to be successful [6]. On the other hand, variation and selection are vital prerequisites for plant breeding program; therefore, having variation among populations and wide amplitude of genetic pool are essential for breeders [7]. From another perspective, since adequate equipment and knowledge for sequencing all plants are not available, analysis and identification of genetic diversity among single plants and populations become important [8]. Assessment of genetic diversity is carried out by several types of markers in plants among which molecular markers have shown advantages over others because of their neutrality and feasibility. Another reason is that they do not depend on age and tissue type and also are not influenced by the environmental conditions [9].

ISSR is one of the most popular markers based on polymerase chain reaction (PCR). This group of molecular markers has been used widely for analysis of genetic diversity among different species of plants [10-17]. Han and Li found 12 polymorph sequences out of 31 in *Peganum harmala* germplasm [18]. Attempts to evaluate genetic variation of Harmal have been very limited thus far. In one report, genetic diversity in coastal and inland desert populations of *Peganum harmala* L. has been evaluated by using Random Amplified Polymorphic DNA (RAPD) technique [19]. In another related study, genetic diversity of *Peganum harmala* was studied based on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [20]. In the current report, we evaluated genetic diversity between 21 Harmal accessions, collected from different regions of Iran using ISSR marker.

Materials and Methods

Plant materials

In this research, seeds of 21 Harmal accessions were collected from the different regions of Iran (Table 1) and then were planted in middle of February in plastic pots kept in the physiology laboratory, campus of agriculture and natural resources, Razi university, Kermanshah, Iran. After 20 days, Harmal young fresh leaves were harvested and stored in -80° in order to be utilized for DNA isolation.

DNA extraction

DNA extraction was carried out based on CTAB method [21]. Purification of isolated DNA was confirmed by 0.8% gel electrophoresis. Finally, DNA samples were stored in 20°C before applying ISSR primers.

ISSR amplification

In this research, 14 ISSR primers were used for screening and exhibiting genetic diversity among all accessions (Table 2). Before doing PCR, 10 ng/µl DNA was produced from stocks based on the results of spectrophotometry analysis. The PCR procedures were performed in a total volume of 25 µl in a "FLEXCYCLER" thermocycler according to Williams *et al.,* [22]. The reaction mixture including 2.5 µl PCR buffer (10mM Tris-Hcl, 50 mM

KCL), 1.2 µl $MgCl_2$ (10 mM), 2.5 µl Primer (10 µM), 0.4 µl mixed dNTPs (0.1mM), 2.5 µl template DNA (5 ng/µl), 0.2 µl DNA *Taq*-polymerase (5U) and 15.7 µl DDW. The number of cycles and their thermal conditions differed for each primer.

Amplified products of PCR operations were run in 1.2% agarose gels with 0.5×TBE and 1 Kbp DNA Ladder. After that, agarose gels were stained with ethidium bromide and visualized under UV 35.

Various degrees of polymorphism were observed by application of primers with high-resolution bands which were used for data analysis.

Statistical analysis

After applying each primer, all amplified bands from all ecotypes were scored for the absence (0) or presence (1) of determined bands. The NTSYS-pc software version 2.02 was used for cluster analysis, performed via Centroid method and Principle coordinate analysis (PCoA).

Table 1. List of 21 Harmal accessions and their place of collection.

code	Place of collection	code	Place of collection
1	Oltan	12	Mashhad
2	Roodsar	13	Qurveh
3	Divandareh	14	Esfarayen
4	Tourkamansahra	15	Naishabour
5	Firoozabad	16	Hashtrood
6	Chalous	17	Lar
7	Meshkinshahr	18	Eshtahard
8	Shahreza	19	Shahrood
9	Kashan	20	Boroojen
10	Bojnoord	21	Sahneh
11	Aghghala		

Table 2. ISSR primers and their amplification results in Harmal germplasm.

NO. ISSR	Primer	Primer sequence	PB	TNB
		5′ → 3′	%	
1	UBC-881	(GGGGT)3G	44.4	9
2	UBC-834	(AG)8TT	80	10
3	UBC-807	(AG)8T	62.50	8
4	UBC-811	(GA)8C	12.5	8
5	UBC-824	(TC)8G	37.5	8
6	UBC-814	(CT)8A	50	8
7	UBC-864	A(CTGA)3CTG	54.54	11
8	UBC-852	(TC)8AA	60	10
9	UBC-876	(GAA)6	85.71	7
10	UBC-822	(TC)8A	70	10
11	UBC-840	(GA)8TT	60	5
12	UBC-826	(AC)8C	66.66	6
13	UBC-868	(GAA)6	75	8
14	UBC-815	(CT)8G	71.44	7

PB: Polymorphic Bonds; **TNB**: Total Number Bonds

Results and Discussion

Primers analysis

All of 14 ISSR primers successfully amplified polymorphism regions at different levels (Table 2). The all ISSR primers amplified 68 polymorphic bands out of 115 (59.13%). The number of bands ranged from five (primer UBC-840) to 11 (primer UBC-864). UBC-876 and UBC-811primers exhibited the highest and lowest percentages of polymorphism by 85% and 12%, respectively. Size of bands varied from 100 to 2500 bps. There is no valid ISSR data on Harmala germplasm in literatures to compare with. Although, El-Bakatoushi *et al.,* used seven RAPD primers

and found 60 (95.24%) polymorphic bands out of total 63 bands across *Peganum harmala* populations [19].

Genetic relationships among accessions and cluster analysis

Jaccard Similarity Matrix (JSM) was performed in order to understand genetic closeness among accessions. The results showed highest similarity between accessions 9 and 14 (collected respectively from Kashan and Esfahan, both located in Esfahan province) by 94%, while the lowest similarity or the highest difference was found between 16 and 18 accessions collected from Hashtrood and Eshtahard respectively (both located in Tehran province) by 55% similarity (Table 3). Cluster analysis based on JSM via Complete method was performed for ISSR binary data in order to understand a genetic relationship among accessions. Complete method was chosen in order to achieve least "chaining effect" [23]. The dendrogram was drown based on complete analysis of the ISSR data (Fig. 1). According to the dendrogram, all 21 accessions were categorized into three groups with about 0.71 distance unit. Accessions 1, 11, 8, 2, 4, 10, 12, 20 and 17 were put in the first group. The second group consisted accessions 3, 9, 14, 6, 5, 13, 19, and 16. Finally, accessions 7 and 18 occupied third group of dendrogram. The cophenetic correlation coefficient was 0.88 that shows a relatively good fit of the data are obtained from the dendrogram. There was not a high compatibility between genetic divergence and geographic origin of accession collection in this research. Bakatoushi *et al.,* also could not find a correlation between genetic diversity and the places of collections for Harmal accessions after using RAPD marker for assessment of genetic variation of this plant in Egypt [19].

Similar results have been reported about other plants such as Groundnut and Shisham [24, 25]. In addition, genetic diversity among all accessions in this study, except Sahneh ecotype, has been carried out based on the seed's protein storage by Rostami-Ahmadvandi *et al.,* and closeness between grouping of accessions based on cluster analysis and their collecting location was not reported [20].

Table 3. Jaccard's similarity matrix of 21 accessions of Harmal.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2	0.72	1.00																		
3	0.86	0.82	1.00																	
4	0.75	0.82	0.85	1.00																
5	0.73	0.83	0.82	0.83	1.00															
6	0.76	0.70	0.83	0.83	0.80	1.00														
7	0.69	0.66	0.69	0.76	0.67	0.71	1.00													
8	0.78	0.75	0.74	0.81	0.72	0.72	0.76	1.00												
9	0.81	0.81	0.91	0.91	0.85	0.89	0.75	0.80	1.00											
10	0.73	0.88	0.83	0.90	0.88	0.81	0.71	0.82	0.89	1.00										
11	0.87	0.76	0.79	0.79	0.76	0.77	0.70	0.82	0.85	0.80	1.00									
12	0.80	0.80	0.82	0.90	0.80	0.80	0.73	0.82	0.85	0.88	0.80	1.00								
13	0.80	0.80	0.87	0.87	0.88	0.88	0.68	0.79	0.92	0.92	0.84	0.88	1.00							
14	0.79	0.79	0.88	0.85	0.86	0.87	0.70	0.75	0.94	0.87	0.83	0.79	0.90	1.00						
15	0.80	0.80	0.90	0.90	0.88	0.85	0.75	0.79	0.92	0.88	0.81	0.92	0.92	0.87	1.00					
16	0.74	0.77	0.83	0.83	0.84	0.78	0.71	0.79	0.85	0.85	0.77	0.81	0.85	0.80	0.88	1.00				
17	0.78	0.78	0.84	0.81	0.75	0.75	0.65	0.73	0.80	0.82	0.78	0.85	0.79	0.77	0.79	0.75	1.00			
18	0.68	0.62	0.65	0.65	0.59	0.66	0.72	0.71	0.68	0.66	0.69	0.69	0.67	0.65	0.64	0.55	0.67	1.00		
19	0.80	0.76	0.82	0.83	0.88	0.84	0.63	0.75	0.85	0.88	0.84	0.88	0.92	0.83	0.88	0.84	0.85	0.62	1.00	
20	0.72	0.79	0.78	0.86	0.80	0.76	0.73	0.75	0.81	0.88	0.76	0.91	0.84	0.75	0.88	0.80	0.81	0.65	0.83	1.00
21	0.73	0.73	0.79	0.83	0.80	0.80	0.67	0.69	0.85	0.80	0.80	0.80	0.84	0.79	0.84	0.81	0.78	0.59	0.88	0.87

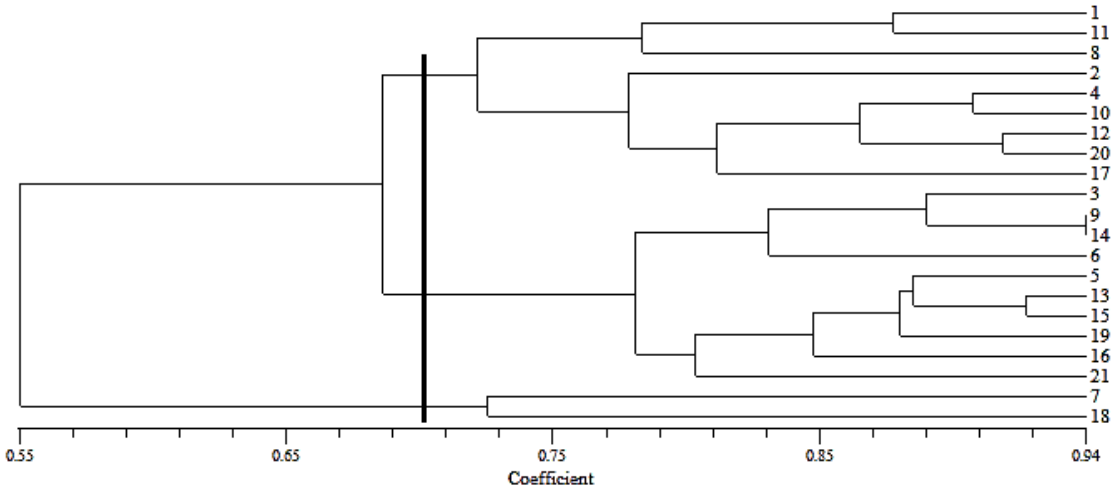

Figure 1. Dendrogram of the relationships among accessions of Harmal by Complete method of cluster analysis.

Principle coordinate analysis

Principle coordinate analysis was performed for ISSR data in order to establish the relationship among accessions and comparision to cluster analysis (Fig. 2). Results showed that distribution pattern of samples was mainly similar to the results from cluster analysis. Similar results were reported by Pezhmanmehr *et al.,* and Rostami- Ahmadvandi *et al*., [16, 20].

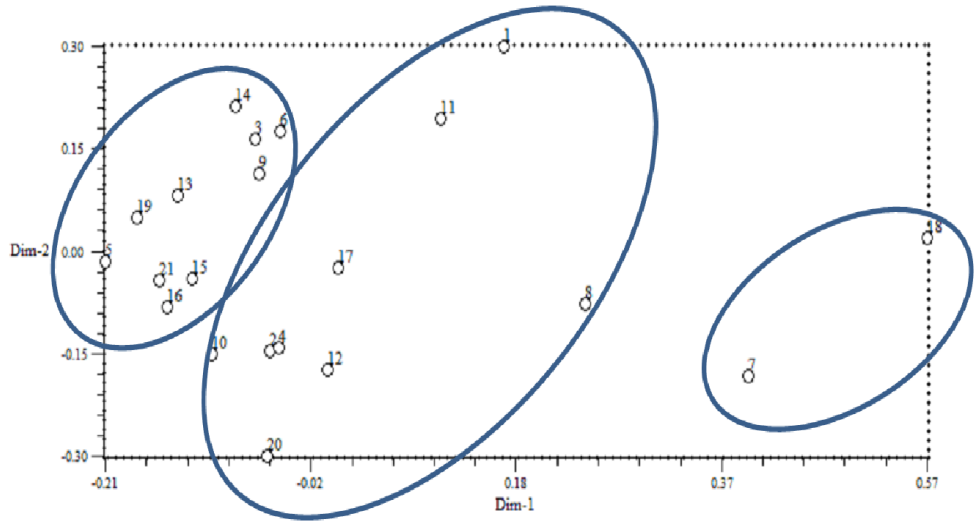

Figure 2. Plot of Harmal accessions by principal coordinate analysis using the Jaccard's similarity coefficients.

Conclusion

The understanding of the genetic diversity is significantly essential not only for the conservation of many species of plants and animals, but also for the maintenance of genetic diversity within the populations. ISSR marker is one of the powerful tools, which has helped in understanding genetic variations through polymorphism of band patterns among different genotypes. In the present study, genetic variation between genotypes was observed based on ISSR marker and according to cluster analysis the genotypes were placed into three groups. The results of this study can be used for future Harmal breeding programs.

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