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Original Article

Genetic Ddiversity Assessment Between Different Populations of *Moringa peregrina* (Forssk.) Fiori and *Moringa oleifera* Lam. in Iran using RAPD, ISSR and R-ISSR Markers.

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Abstract

The present study was conducted to investigate genetic diversity between and within of six populations with different individual numbers of cultivated and non-cultivated provenances of *Moringa peregrina* (Forssk.) Fiori and *Moringa oleifera* Lam. using RAPD, ISSR and various combinations of RAPD and ISSR primers in one multiplex PCR (R-ISSR). 10 primers that produced clear and reproducible fragments after screening of 30 primers were selected for further analysis. A set of 10 primers generated 96 bands ranging in size from 150 to 1600 bp, corresponding to an average of 16 bands per primer and out of which 100 % were polymorphic among 26 individuals. The *PIC* values ranged from 0.16 to 0.31 and *MI* values ranged from 2.16 to 4.65 per primer. The primer R-ISSR (H876+A17) had the highest *PIC* (0.31) and *MI* (4.65) values. A maximum and minimum genetic similarity values were observed between populations (I and V) in *M. oleifera* (0.98) and populations (III and IV) in *M. peregrina* (0.52) respectively. The *Gst* value was 0.7, indicating that 61% of the genetic diversity resided within the populations. Clustering analysis using average algorithm based on Nei's unbiased genetic distance, classified the *Moringa* Adans. populations into five major groups. The PCOA data confirmed the results of clustering. The results of this study revealed that R-ISSR markers could be efficiently used for genetic differentiation of the *Moringa* individuals. The primers used in this article are useful to detection of a high level of polymorphism and it can be used to guide future breeding studies and management of *Moringa* genus.

Keywords: *Moringa*, Genetic diversity, RAPD, ISSR and R-ISSR.

Introduction

Moringa genus is one of the most important medicinal plants belongs to the Moringaceae family which about 31 species have been identified in the world (IPNI). Iran is one of the most important sources of *Moringa peregrina* species in the world. It is one of the most economically important crops widely cultivated in many area of the world for its medicinal, nutritional values and also for bio-fuel resource. It is a suitable tree for cultivation in arid area due to its tolerance to inert stresses [1]. The most famous *Moringa* species are *Moringa oleifera* and *Moringa peregrina* [2].

M. peregrina is a green deciduous tree with sweet and oil rich white seeds. It is distributed in tropical and non- tropical regions. Iran, Egypt, Sudan, Palestine, the red sea region, Jordan and Ethiopia to Somalia are the main centers of distribution [2]. *M. peregrina* is an endangered plant due to slow

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regeneration rate after browsing and unmanaged grazing and also it is multipurpose tree with a wide variety of potential uses including its wood is used in building on its resistance to termites, its seeds' oil is used to make aroma and its medicinal activity such as skin protection, laxative and ventral pain, [3,4]. *M. oleifera* is an evergreen tree native to tropical Africa and is also widely distributed in Ceylon, India, Sudan, Madagascar and south of Iran [5]. It has been cultivated in tropical regions all over the world for its features such as medicinal uses, high value of nutrition suitable for livestock and humans, high oil's seed which is edible and high content of mineral, vitamins and proteins in whole plants [6,7]. Narrow information is available on the genetic diversity of *M. oleifera* and *M. peregrina* in Asia and Africa especially in Iran. Genotypic characteristics using molecular markers to assess genetic diversity has become a model to design effective sampling strategies for future breeding programs [8-15].The only available report on the molecular study of *M. peregrina* showed its genetic stability using ITS analysis [15].The loss of plant genetic resources, particularly those of wild species, has led to the development of *ex-situ* conservation methods to maintain genetic diversity. The objective of this research therefore, was to assess the genetic diversity within some cultivated *M. oleifera* and some endemic plants of *M. peregrina* from South of Iran to see if there is any genetic relatedness as well as to evaluate 10 arbitrary RAPD, ISSR, and R-ISSR primers for genetic diversity assessment. The information obtained from this study will be used to develop effective strategies for formation, conservation and utilization of third generation seed orchard of *Moringa* genetic resources in Iran.

Material and Methods

Plant Materials and DNA Extraction

Moringa samples were surveyed and young leaves were collected from trees growing in different locations representing six districts of Hormozgan, Sistan and Baluchestan provinces in Iran (Table1).Young fresh leaves immediately placed on ice in a cooler, and transferred to the lab in Tehran (RIFR).

Total DNA was extracted following the major modified CTAB method [16]. Approximately 0.4 gr. leaves was ground to a fine powder in a mortar with liquid nitrogen and then transferred to a 2 ml Eppendorf tube filled with 800*µl* of 2% CTAB extraction buffer(100 mM Tris –HCl pH=8, 25 mM EDTA pH=8, 2M NaCl, 0.3% mercaptoethanol and 1.5% PVP40) after being incubated at 65◦C for 90 min and each sample mixed thoroughly by inversion every 10min and then about 900*µl* of chloroform-isoamylalcohol (24:1) was added to the solution and mixed well for 10min and centrifuged at 10000rpm for 15min. The supernatant was then transferred into a new tube and then added 70% Cold isopropanol about 0.7 of total volume of supernatant collected and incubate for 12h at -20 °C and then centrifuged at 12000rpm for 13min. DNA pellets were washed twice with 70% ethanol, allowed to dry by concentrator at 25 °C for 12min and dissolved pellets in 300*µl* TE (10:1) buffer and then 5*µl* RNaseA were added, and mixed well and incubate at 37◦C for 1h. The homogenate was then extracted with 300 *µl* of phenol/chloroform/isoamyl alcohol (25:24:1) and was centrifuged at 12,000 rpm for 10min, the supernatant was transferred into a new tube and were added 30-40*µl* sodium acetate 3M and 400-600*µl* cold pure ethanol and incubate at -20°C for 30 min and then centrifuged at 13000rpm for 10min. DNA pellets were washed with 70% ethanol, allowed to dry by concentrator and dissolved in 250*µl* TE (10:0.1)buffer and was stored at -80 °C.

DNA Quantity and Quality

Purified DNA was quantified using a BioQuest^{TM} CE2502 Spectrophotometer (CECIL Instrument Limited Milton Technical Center, Cambridge, England). DNA concentration is estimated by measuring the absorbance at A230, A260, A280 and A320. A pure sample of DNA has the ratio (A260/A280) at 1.8 and is relatively free from protein pollution. The purified total DNA was qualified by 0.8% agarose gel electrophoresis and the DNA samples were diluted to 10ng/µl and stored at -20 ºC.

Primers and Assays

Primers were purchased in lyophilized form from TIB MOLBIOL-Germany, totally 30 primers were tested to amplify DNA and among them, four pairs and two single primers with considerable polymorphism and reproducibility were selected for further analysis (Table 2). PCR were performed in 50*µl* volume consisted of 1X PCR buffer, 1mM $MgCl_2$, 0.2 mM each of dNTPs, 0.5 µM primer, 2.5U of Taq DNA polymerase (Qiagen-Germany) and 14*ng* of template DNA. Amplification was

performed in a Eppendorf Mastercycler gradient Thermal Cycler under the following conditions: 5 min at 95 ºC for 1 cycle, followed by 1 min at 95 ºC, 1 min at 27.7 ºC, 36 ºC or 38 ºC (Depending on the primer), and 1.5 min at 72 ºC for 40 cycles, and 10 min at 72 ºC for a final extension. Amplification products were separated on 1% agarose gels run at 50 V in 1×TBE, visualized by staining with ethidium bromide, and photographed ultraviolet light. Molecular weights were estimated using a 100 bp DNA ladder.

Data Analysis

Marker indices (*MI*) were estimated for the R-ISSR, ISSR and RAPD markers to characterize the capacity of each primer to detect polymorphic loci among the populations and individuals [17]. The *PIC* value was calculated using the formula *PICi=2fi (1-fi)*, as proposed by Roldan-Ruiz, *et al*. (2000) [18]. The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the R-ISSR, ISSR and RAPD and assembled in a data matrix. POPGENE software 1.31 [19] and GenAlex [20] were utilized to produced the single population gene frequencies and the grouped population gene frequencies as well as Nei's [21] genetic distances matrix between the populations from the 0, 1, data matrix. The results of distance matrix used to construct a (Average) phenogram for the six populations using JMP software [22]. Also observed number of alleles (*na*); Number of effective alleles (*ne*); Nei's [23] genetic diversity index(*h*); Shannon's Information index (*I*); the total Heterozygosity (*Ht*); the Expected Heterozygosity within subpopulations (*Hs*); the coefficient of genetic differentiation (*Gst*) and estimate of gene flow from *Gst* (*Nm*) were analyzed with POPGENE and GenAlex software's. The R-ISSR, ISSR and RAPD data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier [24].The analysis of AMOVA was performed using GenALEx software. GenAlex was also used to calculate a Principal Coordinates Analysis (PCOA) that plots the relationship between distance matrix elements based on their first two principal coordinates.

Results and Discussion

Random Amplified Polymorphic DNA (RAPD) Analysis:

In this marker one of the advantages is the requirement for only small amounts of DNA (10-100 ng per reaction). Some researchers such as Mgendi, *et al*. (2010) [9] and Cruz da Silva *et al*. (2012)[10] applied RAPD markers to assess the genetic diversity of *Moringa* accessions. The results showed that conservation strategies should be adopted for these plants. In this study RAPD assays were performed with many random primers. Of these, three primers namely (A17, U23 and D01) (Table 2) produce polymorphic and reproducible bands alone or with ISSR markers. These primers were selected for further screening. In an assay of the U23 primer as a RAPD marker after three times repeat on the 26 accessions, 22 bands were scored in the size range 280-1600bp. Of the 22 bands, 22 bands were polymorphic (Table 3), with 100% polymorphisms. Polymorphism information content (*PIC*) of six populations for this marker separately were (0.137, 0.098, 0, 0, 0.038 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.248, 0.153, 0, 0, 0.604 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals.

Inter-Simple Sequence Repeats (ISSR) Analysis:

ISSR is an effective, cost efficient method for genotype identification and assessment of genetic relationships within and among populations. For present investigation ISSR assay were performed with many random primers. Of these, four primers namely (H808, H834, H844 and H876) (Table 2) produce polymorphic and reproducible bands alone or with RAPD markers. These primers were selected for further screening. In an assay of the H808 primer as an ISSR marker after three times repeat on the 26 accessions, amplified a total of 12 scorable bands ranging in size from 150 – 900bp. Of the 12 bands, 12 bands were polymorphic (Table 3), with 100% polymorphisms. Polymorphism information content of six populations for this marker separately were (0.141, 0.128, 0, 0, 0.069 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.238, 0.218, 0, 0, 0.101 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals.

R-ISSR: Inter-simple Sequence Repeats (ISSR) and Random-amplified Polymorphic DNA (RAPD) Analysis:

The combined use of RAPD and ISSR primers in the same polymerase chain reaction (PCR) reactions reveal new genomic loci that could not be detected with either technique alone. For present investigation R-ISSR assay were performed with many random primers. Of these, four pairs primer namely (H808 & U23, H834 & U23, H844 & D01 and H876 & A17) (Table 2) produce polymorphic and reproducible bands. These pairs primer were selected for further screening. In an assay of the four pairs primer as R-ISSR markers after three times repeat on the 26 accessions, respectively amplified a total of (13, 15, 19 and 15) scorable bands ranging in size from 150 – 1500, 350-1250, 400-1600, 480-1600 respectively (Table 3). Of the 13 bands obtained from the first pairs (H808 & U23), 13 bands were polymorphic. Polymorphism information content of six populations for this marker separately were (0.168, 0.133, 0, 0, 0.96 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.253, 0.241, 0, 0, 0.139 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals. Of the 15 bands obtained from the second pairs (H834 & U23), 15 bands were polymorphic. Polymorphism information content of six populations for this marker separately were (0.197, 0.207, 0, 0, 0.193 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.301, 0.328, 0, 0, 0.282 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals. Of the 19 bands obtained from the third pairs (H844 & D01), 19 bands were polymorphic. Polymorphism information content of six populations for this marker separately were (0.153, 0.096, 0, 0, 0.109 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.275, 0.173, 0, 0, 0.159 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals. Of the 15 bands obtained from the fourth pairs (H876 & A17), 15 bands were polymorphic. Polymorphism information content of six populations for this marker separately were (0.335, 0.250, 0, 0, 0.110 and 0) respectively. Also the Shannon's information index (*I*) of six populations for this marker separately were (0.507, 0.376, 0, 0, 0.161 and 0) respectively. The (*PIC*) and (*I*) values for each population showed that three out of six populations have genetically identical individuals.

Combined Marker (ISSR, RAPD and R-ISSR) Analysis:

Six populations (26 individuals) were analyzed using ISSR, RAPD and R-ISSR primers. Six selected primers amplified a total of 96 scorable bands ranging in size from 150 to 1600 bp, corresponding to an average of 16 bands per primer (Table 3 and Fig. 1). The highest number of bands was scored for population I (*M. oleifera*) of which 78.12 % were polymorphic, followed by population II (*M. peregrina*) with 53.12 % polymorphic bands. The least polymorphism (23.96%) was observed in population V (*M. oleifera*). Populations (III, IV and VI) haven't any polymorphism bands. This suggests that individuals within these populations have been genetically identical. *PIC* value ranged from 0.16 to 0.31 and *MI* value ranged from 2.16 to 4.65 per primer. The primer R-ISSR (H876 & A17) had the highest PIC (0.31) and MI (4.65) values (Table 3). The total mean values of gene diversity (*h*) and Shannon's Information index (*I*) of the 26 individuals of different populations for six(ISSR ,RAPD and R-ISSR) markers determined were 0.216± 0.129 and 0.357± 0.174 respectively (Table 4). The highest mean value of (*h*) and (*I*) of the 26 individuals was found for (H834 & U23, H876 & A17) R- ISSR marker (0.5±0.12 and 0.69±0.14).

Among six populations, just population I (*M. oleifera*) had the highest *h* and *I* values (0.185±0.155 and 0.30±0.22) for six selected markers. The mean value of parameters such as (*ne*), (*I*), (*h*), (*Ht*), (*Hs*), (*Gst*) and (*Nm*) based on data of six selected markers were (1.31±024),(0.22±129),(0.357±0.174),(0.218±0.022), (0.072±0.003), 0.671 and 0.245 respectively, while Loci of R-ISSR markers such as (H834 & U23 –E, H876 & A17-F, and J) showed the highest (*ne*), (*I*), (*h*), (*Ht*) and (*Hs*) values (Table 4). The amount of gene flow among 96 scorable bands, estimated as *Nm* was found to be ranges from zero to 9.9 (Table 4).

Nei's (1987) [25] unbiased genetic identity and genetic distance for six primers (Table 2) data determined among the *Moringa* populations are given in Table 5.

Table 1 A listing of 26 *Moringa* Adans. accessions sampled from three provinces of Iran

Populations	Code No.	Species	Latitude	Longitude	Location(Province)
I	1	*M. oleifera*	27 12 7.0	60 30 4.7	Bampoor(Sistan)
	2	*M. oleifera*	27 12 7.4	60 30 4.2	Bampoor(Sistan)
	3	*M. oleifera*	27 12 7.5	60 30 3.7	Bampoor(Sistan)
	4	*M. oleifera*	27 12 7.4	60 30 4.2	Bampoor(Sistan)
	5	*M. oleifera*	27 12 7.4	60 30 4.7	Bampoor(Sistan)
	6	*M. oleifera*	27 12 7.2	60 30 4.4	Bampoor(Sistan)
	7	*M. oleifera*	27 12 7.7	60 30 4.9	Bampoor(Sistan)
	8	*M. oleifera*	27 12 7.3	60 30 3.9	Bampoor(Sistan)
	9	*M. oleifera*	27 12 7.4	60 30 3.9	Bampoor(Sistan)
II	16	*M. peregrina*	27 12 4.6	60 30 2.7	Bampoor(Sistan)
	17	*M. peregrina*	27 12 2.2	60 30 3.0	Bampoor(Sistan)
	18	*M. peregrina*	27 12 1.0	60 30 1.9	Bampoor(Sistan)
	19	*M. peregrina*	27 12 7.0	60 30 2.9	Bampoor(Sistan)
	20	*M. peregrina*	27 12 2.0	60 30 2.6	Bampoor(Sistan)
	21	*M. peregrina*	27 12 1.2	60 30 3.2	Bampoor(Sistan)
III	10	*M. peregrina*	26 18 41.3	60 10 6.7	Keneshki(Baluchestan)
	11	*M. peregrina*	26 18 41.9	60 10 7.1	Keneshki(Baluchestan)
	12	*M. peregrina*	26 18 41.6	60 10 6.9	Keneshki(Baluchestan)
IV	13	*M. peregrina*	26 18 43.1	60 21 4.1	Keshik(Baluchestan)
	14	*M. peregrina*	26 18 43.4	60 21 4.6	Keshik(Baluchestan)
	15	*M. peregrina*	26 18 43.6	60 21 4.3	Keshik(Baluchestan)
V	22	*M. oleifera*	28 13 27	55 33 27	Kahurestan(Hormozgan)
	23	*M. oleifera*	28 13 22	55 33 25	Kahurestan(Hormozgan)
VI	24	*M. peregrina*	28 13 27	55 33 27	Kahurestan(Hormozgan)
	25	*M. peregrina*	28 13 21	55 33 32	Kahurestan(Hormozgan)
	26	*M. peregrina*	28 13 23	55 33 35	Kahurestan(Hormozgan)

Table 2 RISSR, ISSR and RAPD primers with their sequences and annealing temperature (TM) (R=A/G, Y=C/T)

Primer	Primer Code	)5'-3'(Sequence	)◦C(Annealing Temp.
P(1)	H876	$(GATA)_2(GACA)_2$	27.7
	A17	GACCGCTTGT	27.7
P(2)	H808	$(AG)_8C$	38
	U23	CCCGCCTTCC	38
P(3)	H834	$(AG)_8YT$	38
	U23	CCCGCCTTCC	38
P(4)	H844	$(CT)_8RC$	36
	D01	ACCGCGAAGG	36
P(5)	U23	CCCGCCTTCC	38
P(6)	H808	$(AG)_8C$	38

Table 3 ISSR, RAPD and RISSR primers successfully used in this study and the number of total and polymorphic bands amplified in six populations with Polymorphism information content (PIC) and marker index (MI) values. Y= C /T, R = A /G

Band amplitude produced	MI	PIC	Percentage of polymorphism	Number of polymorphic bands	Total number of bands	Sequence (5' -3')	primer
150-900	2.16	0.18	100	12	12	$(AG)_8C$	H808
280-1600	3.74	0.17	100	22	22	CCCGCCTTCC	U23
150-1500	2.73	0.21	100	13	13	$(AG)_8C$ CCCGCCTTCC	H808 & U23
350-1250	3.9	0.26	100	15	15	$(AG)_8YT$ CCCGCCTTCC	H834 & U23
400-1600	3.04	0.16	100	19	19	$(CT)_8RC$ ACCGCGAAGG	H844 & D01
480-1600	4.65	0.31	100	15	15	$(GATA)_2(GACA)_2$ GACCGCTTGT	H876 & A17

Table 4 Overall genetic variability across six populations with 26 individuals. Nm: estimate of gene flow from Gst, Nm = 0.5 (1 -Gst)/Gst. Total Heterozygosity (Ht), Expected Heterozygosity within subpopulations (Hs), coefficient of genetic differentiation (Gst)

Locus	Observed No. of alleles	Effective No. of alleles	Nei's gene Diversity(h)	Shannon's information Index (I)	Ht	Hs	Gst	Nm
H808-A	2	1.04	0.039	0.098	0.03	0.03	0.07	6.29
H808-B	2	1.04	0.039	0.098	0.03	0.03	0.07	6.29
H808-C	2	1.13	0.11	0.23	0.13	0.11	0.16	2.63
H808-D	2	1.46	0.31	0.49	0.34	0.08	0.75	0.16
H808-E	2	1.65	0.39	0.58	0.38	0.12	0.67	0.24
H808-F	2	1.35	0.26	0.43	0.30	0.03	0.88	0.06
H808-G	2	1.04	0.03	0.09	0.03	0.03	0.07	6.29
H808-H	2	1.13	0.11	0.23	0.08	0.07	0.12	3.73
H808-I	2	1.04	0.03	0.09	0.02	0.02	0.05	9.90
H808-J	2	1.31	0.23	0.4	0.33	0.07	0.79	0.13
H808-K	2	1.35	0.26	0.43	0.30	0.03	0.88	0.06
H808-L	2	1.41	0.29	0.46	0.32	0.05	0.84	0.09
H808U-A	2	1.08	0.08	0.17	0.06	0.05	0.16	2.67
H808U-B	2	1.3	0.23	0.39	0.29	0.03	0.91	0.05

H808U-C	2	1.65	0.39	0.58	0.29	0.16	0.41	0.72
H808U-D	2	1.35	0.26	0.43	0.30	0.04	0.85	0.08
H808U-E	2	1.38	0.27	0.45	0.15	0.10	0.30	1.13
H808U-F	2	1.04	0.03	0.09	0.03	0.03	0.07	6.29
H808U-G	2	1.25	0.2	0.35	0.1	0.07	0.29	1.2
H808U-H	2	1.32	0.11	0.23	0.13	0.11	0.16	2.63
H808U-I	2	1.25	0.2	0.35	0.28	0	1	0
H808U-J	2	1.38	0.27	0.45	0.15	0.1	0.3	1.13
H808U-K	2	1.3	0.23	0.39	0.3	0.03	0.91	0.05
H808U-L	2	1.25	0.2	0.35	0.28	0	1	0
H808U-M	2	1.41	0.29	0.46	0.36	0.11	0.68	0.22
H834U-A	2	1.04	0.03	0.09	0.03	0.03	0.07	6.29
H834U-B	2	1.08	0.08	0.17	0.12	0.09	0.19	2.07
H834U-C	2	1.31	0.23	0.4	0.16	0.12	0.29	1.21
H834U-D	2	1.58	0.36	0.55	0.4	0.17	0.58	0.36
H834U-E	2	1.99	0.5	0.69	0.499	0.23	0.54	0.43
H834U-F	2	1.13	0.11	0.23	0.13	0.1	0.19	2.17
H834U-G	2	1.36	0.26	0.43	0.32	0.05	0.84	0.09
H834U-H	2	1.36	0.26	0.43	0.35	0.09	0.73	0.18
H834U-I	2	1.55	0.35	0.54	0.34	0.07	0.78	0.14
H834U-J	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
H834U-K	2	1.08	0.08	0.17	0.06	0.05	0.16	2.67
H834U-L	2	1.93	0.48	0.67	0.5	0.19	0.61	0.31
H834U-M	2	1.53	0.34	0.53	0.35	0.09	0.74	0.17
H834U-N	2	1.08	0.08	0.17	0.06	0.05	0.15	2.67
H834U-O	2	1.81	0.44	0.64	0.49	0.13	0.73	0.18
H844D-A	2	1.04	0.03	0.09	0.02	0.02	0.05	9.9
H844D-B	2	1.08	0.07	0.17	0.04	0.03	0.1	4.5
H844D-C	2	1.55	0.35	0.54	0.46	0.03	0.92	0.04
H844D-D	2	1.35	0.26	0.43	0.3	0.03	0.88	0.06
H844D-E	2	1.04	0.03	0.09	0.02	0.02	0.05	9.9
H844D-F	2	1.13	0.12	0.24	0.06	0.05	0.16	2.67
H844D-G	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
H844D-H	2	1.13	0.12	0.24	0.06	0.05	0.15	2.67
H844D-I	2	1.3	0.23	0.4	0.29	0.02	0.94	0.03
H844D-J	2	1.47	0.32	0.5	0.37	0.13	0.65	0.27
H844D-K	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
H844D-L	2	1.19	0.16	0.3	0.1	0.09	0.2	1.9
H844D-M	2	1.5	0.32	0.5	0.37	0.12	0.68	0.24
H844D-N	2	1.2	0.16	0.3	0.18	0.14	0.22	1.81
H844D-O	2	1.36	0.26	0.43	0.35	0.09	0.75	0.17
H844D-P	2	1.19	0.16	0.3	0.1	0.09	0.2	1.9
H844D-Q	2	1.08	0.08	0.17	0.04	0.03	0.1	4.5
H844D-R	2	1.09	0.08	0.17	0.1	0.09	0.2	1.9
H844D-S	2	1.42	0.3	0.5	0.34	0.07	0.8	0.13
H876A-A	2	1.53	0.34	0.53	0.4	0.16	0.6	0.32
H876A-B	2	1.5	0.32	0.5	0.37	0.12	0.68	0.24
H876A-C	2	1.33	0.25	0.42	0.13	0.08	0.38	0.82
H876A-D	2	1.3	0.23	0.4	0.15	0.12	0.19	2.19
H876A-E	2	1.36	0.27	0.44	0.2	0.17	0.19	2.13
H876A-F	2	1.98	0.496	0.689	0.499	0.18	0.64	0.28
H876A-G	2	1.6	0.38	0.57	0.38	0.12	0.7	0.22
H876A-H	2	1.6	0.37	0.56	0.37	0.12	0.68	0.24
H876A-I	2	1.33	0.25	0.42	0.13	0.08	0.38	0.81
H876A-J	2	1.97	0.493	0.686	0.496	0.15	0.69	0.23
H876A-K	2	1.26	0.2	0.36	0.1	0.07	0.29	1.2
H876A-L	2	1.83	0.45	0.65	0.48	0.1	0.79	0.13
H876A-M	2	1.36	0.26	0.43	0.16	0.13	0.2	1.97

H876A-N	2	1.47	0.32	0.5	0.33	0.07	0.77	0.15
H876A-O	2	1.13	0.11	0.23	0.07	0.06	0.07	6.33
U23-A	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
U23-B	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
U23-C	2	1.36	0.26	0.43	0.3	0.03	0.88	0.06
U23-D	2	1.26	0.2	0.36	0.28	0	1	0
U23-E	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
U23-F	2	1.09	0.08	0.17	0.06	0.05	0.16	2.67
U23-G	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
U23-H	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
U23-I	2	1.19	0.16	0.3	0.15	0.12	0.19	2.19
U23-J	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
U23-K	2	1.53	0.35	0.53	0.36	0.1	0.71	0.2
U23-L	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
U23-M	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
U23-N	2	1.18	0.15	0.29	0.16	0.14	0.15	2.73
U23-O	2	1.6	0.37	0.56	0.37	0.12	0.68	0.24
U23-P	2	1.65	0.39	0.58	0.38	0.13	0.65	0.26
U23-Q	2	1.04	0.04	0.1	0.03	0.03	0.07	6.3
U23-R	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
U23-S	2	1.35	0.26	0.43	0.3	0.04	0.85	0.08
U23-T	2	1.36	0.26	0.43	0.3	0.03	0.88	0.06
U23-U	2	1.3	0.23	0.39	0.29	0.02	0.94	0.03
U23-V	2	1.04	0.04	0.1	0.02	0.02	0.05	9.9
Mean	2	1.31	0.216	0.357	0.218	0.072	0.67	0.24
St.Dev.	-	0.24	0.129	0.174	0.022	0.003	-	-

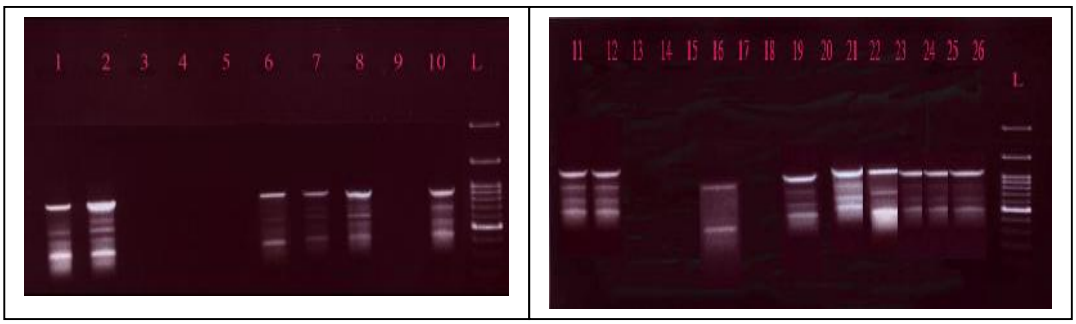
Table 5 Nei's Unbiased Original Measures of Genetic Identity and Genetic distance. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Population	I (*M. oleifera*)	II (*M. peregrina*)	III (*M. peregrina*)	IV (*M. peregrina*)	V (*M. oleifera*)	VI (*M. peregrina*)
I	0	0.936	0.763	0.851	0.980	0.868
II	0.064	0	0.746	0.868	0.929	0.878
III	0.237	0.254	0	0.594	0.740	0.646
IV	0.149	0.132	0.406	0	0.879	0.760
V	0.020	0.071	0.260	0.121	0	0.872
VI	0.132	0.122	0.354	0.240	0.128	0

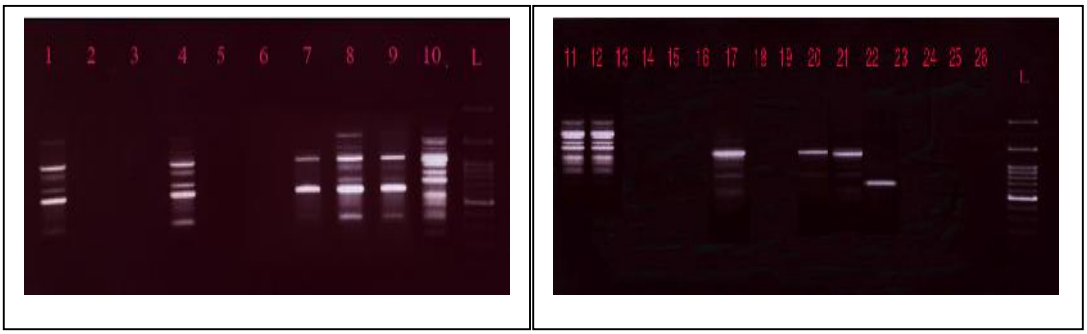
Table 6 Analysis of molecular variance (AMOVA) for six populations of two *Moringa* Adans. species based on ISSR, RAPD and R-ISSR markers.

Sources	Degrees of freedom (df)	Sum of squared (SS)	Mean square (MS)	Variance components	Percentage of variance
Among Populations	5	163.12	32.62**	5.82	39
Within Populations	20	179.11	8.96**	8.96	61
Total	25	342.23	-	14.78	100

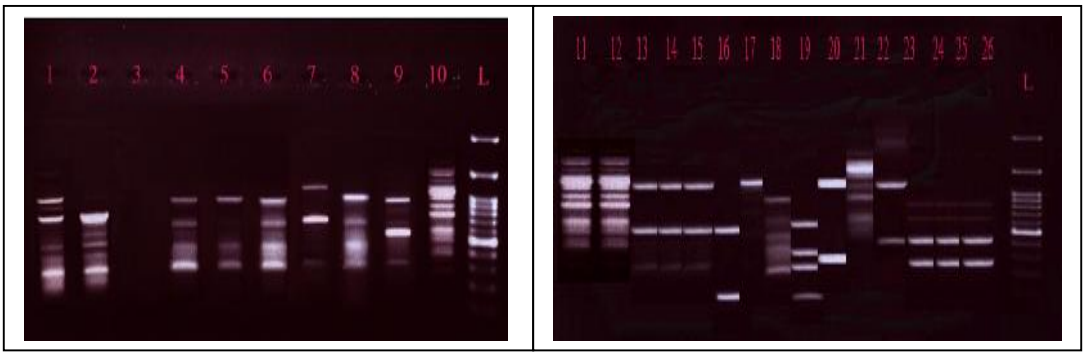
**. Significant at 1% level of probability



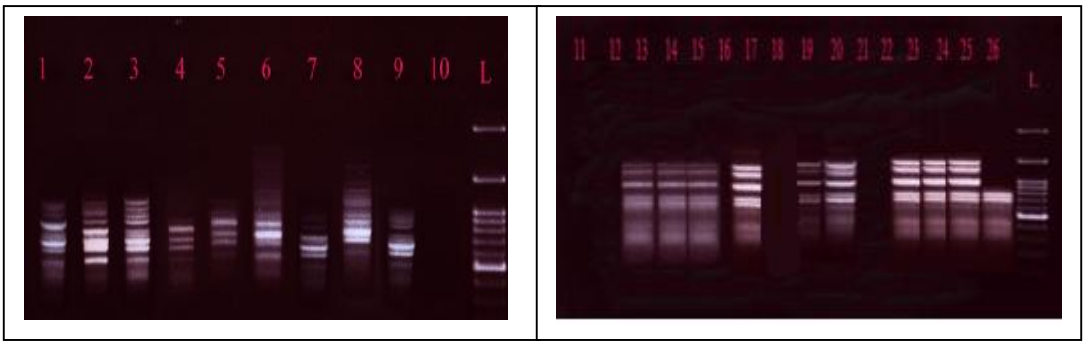
ISSR *(a)*: profile for 26 individuals (according Table 1) produced using primer H808.



RAPD *(b)*: profile for 26 individuals (according Table 1) produced using primer U23.



R-ISSR*(c)*: profile for 26 individuals (according Table 1) produced using primer (H808 & U23).



R-ISSR(c): profile for 26 individuals (according Table 1) produced using primer (H876 & A17).

Fig. 1 ISSR *(a)*, RAPD*(* b) and R-ISSR *(c)* marker profiles of the six populations of *Moringa* Adans. species generated by primers H808, U23, (H808 & U23) and (H876 & A17) in 1% agarose gel. Lane (L): 100bp DNA ladder.

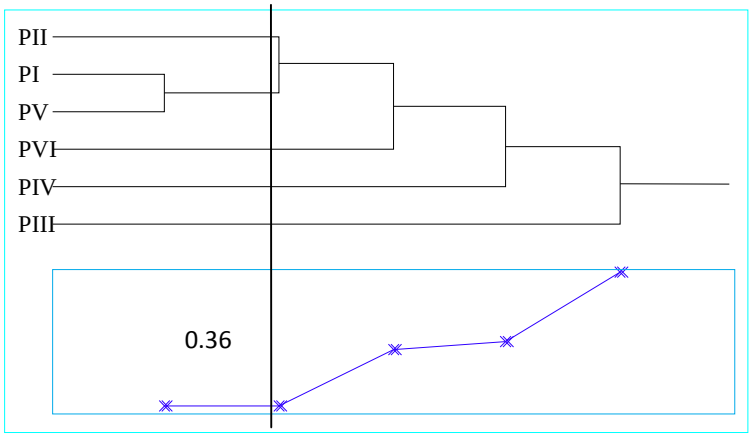


Fig. 2 Dendrogram constructed from Nei's unbiased genetic distance matrix among six populations, clustered with the Average method (r=0.92) based on ISSR, RAPD and R-ISSR data.

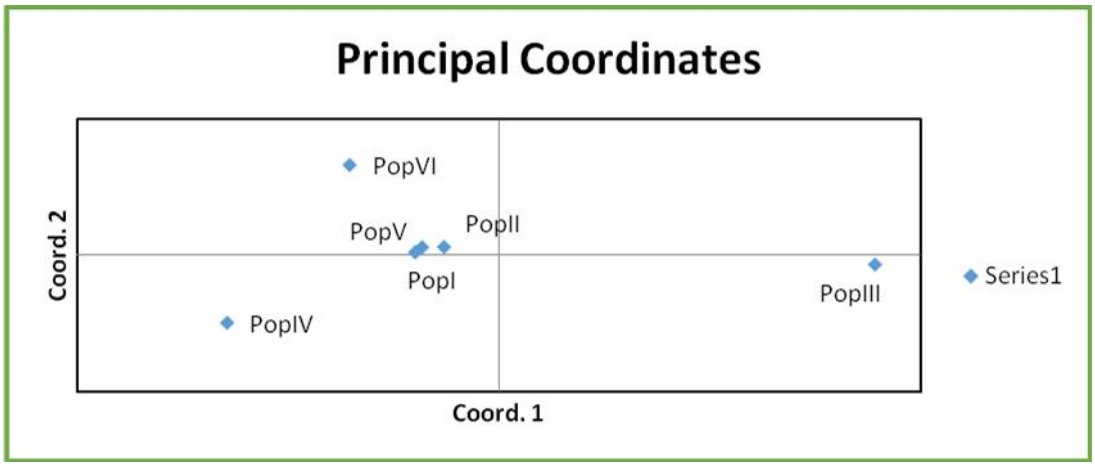


Fig. 3 Scatter plot of six populations for the first two PCOA analyses

The value of genetic identity varied from 0.646 between *M. peregrina* (Hormozgan) and *M. peregrina* (Baluchestan) to 0.980 between *M. oleifera* (Sistan) and *M. oleifera* (Baluchestan). The (*Gst*) value was 0.671, indicating that 61% of the genetic diversity resided within the populations. Clustering analysis using Average algorithm based on Nei's Unbiased Measures of Genetic distance, classified the *Moringa* populations into five major groups. The cophenetic correlation of Average tree was about (r=0.92). The rate of genetic affinities and relatedness of the *Moringa* taxon under consideration can also be observed between the six populations (Fig. 2). Four clusters, consisting of *M. peregrina* from different areas (Table 1) can be observed separately. The results of AMOVA analysis [24] showed that 39% and 61 % of genetic diversity resided between and within the populations respectively and genetic variation among and within populations were significant at 1% level (Table 6). PCOA analysis showed that the first three factors comprised exactly 100 % of total variance when the first, second and third axis comprised 77.37, 17.14 and 5.49 % of total variance respectively. Average dendrogram clustering of ISSR, RAPD and R-ISSR data produced similar results supported by PCOA ordination plot (Fig. 2 and 3). The results of this study revealed that some adjusted primers in R-ISSR markers could be efficiently used for genetic differentiation of the *Moringa* accessions. The primer (H876 & A17) is useful to detection of a high level of polymorphism and it can be used to guide future breeding studies and management of third generation seed orchard of *Moringa* genetic resources in Iran.

Discussion

The present study provides the first report on genetic stability of *M. peregrina* and *M. oleifera* using R-ISSR analysis in Iran. The experimental

results of this study will afford evidence for the reliability and usefulness of R-ISSR markers, to estimate genetic diversity within and between *Moringa* populations.

The genome of *M. oleifera* and *M. peregrina* has not been sequenced yet, but advances in genomic research have published new tools, such as molecular markers, that have become necessary for crop improvements. This study focused on the comparative evaluation of different PCR-based molecular markers (RAPD, ISSR and R-ISSR) and their potential use for fingerprinting and assessing the genetic diversity among accessions of *M. oleifera* and *M. peregrina* species. Comparative studies in *Moringa* genus and major oilseeds crops involving RAPD, SSR, ISSR, AFLP markers have been successfully used by various researchers [8-15 and 26-30). The results of SSR studies on *M. oleifera* and *M. peregrina* grown in Saudi Arabia revealed 6/9 primers only produced bands in *M. peregrina* genotypes [30]. Also, Ganesan, *et al.* [12] study on *Moringa oleifera* using SSR markers who reported that gene diversity range between 0.01 and 0.49 with an average of 0.18. It is clearly seen that SSR markers produce the least number of bands. The result of RAPD studies on *M. oleifera* exchanged germplasm of University of Florida by Cruz da Silva, *et al.* [10] detected low diversity in that investigation and suggested new activities of collection should be realized, with integration and characterization of new accessions to ensure the ever increasing diversity of the collection. The results of AFLP analysis of genetic variation in *M. oleifera* showed that there are significant differences between regions and populations, even though out- crossing perennial plants is expected to maintain most variation within populations [8]. The results of investigation Saini, *et al*. [13] on genetic diversity of commercially grown *M. oleifera* cultivar from India using RAPD and ISSR and cytochrome P_{450}-based markers suggested that ISSR markers are the most effective for assessment of genetic diversity and there are high genetic diversity among cultivars with low distinction between geographical origins. In this study the RAPD primer generated higher number of polymorphic bands compared to other primers, but R-ISSR primers produced efficient separation bands on an agarose gel and agree with the study conducted by [19, 31, and 32].

In this research, populations (I and II) had high value of (*I*) index with all primers but this value was the highest in R-ISSR marker in compare with other markers. High level of genetic polymorphism detected by some of these markers is in agreement with the assumption that out-breeding plant species from natural population will have higher level of genetic diversity than, when compared to in-breeding plant species. High level of genetic differentiation in all populations except populations (I and V) as reflected by the genetic diversity parameters, such as (*Hs*), percentage polymorphism, (*I*) index and others are pointing to the fact that there is wild variability in these populations of *Moringa*. Additionally, interaction between various ecological and biological factors, such as selection, gene flow, genetic drift, and mating system, affects the genetic structure of any plant populations. This subject is very important for successful crossing and improvement programs in formation, conservation and utilization of third generation seed orchard of *Moringa* genetic resources in Iran. The overall genetic variability and differentiation pattern observed in *M. oleifera* and *M. peregrina* populations are in agreement with those of other open pollination plant species [27].The value of average percentage of polymorphism (100%) in all primers selected in this study almost was found to be dissimilar to earlier studies of Saini *et al*. [13].The highest *MI* produced by R-ISSR (H876 & A17) (4.65) marker system was due to its Polymorphism information content value (0.31).

The cluster analysis of six populations of *M. oleifera* and *M. peregrina* based on Average method suggested the formation of two separate species and only for *M. peregrina* accessions from different ecological locations, four separate clusters formed at different genetic distances. Clustering of individuals from the same population in different clusters indicates high genetic variation within populations which may be attributed to the use of seed sources or breeding system which is in agreement with the fact that it is predominantly an out-crossed plant. The *M. oleifera* species collected from “Bampoor in Sistan” and “Kahurestan in Hormozgan” regions found in one cluster, this further supports the idea that spread of planting materials has taken place in the form of cuttings, seed and/or high rates of gene flow between the adjacent populations. The *M. peregrina* species collected from each provenance of Sistan, Baluchestan and Hormozgan provinces found in the separate clusters that could potentially be used

in breeding efforts and conserving germplasms as they are found to be genetically distinct.

In conclusion, the results from this research have shown that enough variability and genetic heritability exist in the studied characters among the evaluated 15 individuals of four populations of *M. peregrina* species. These observations indicate major diversity exists between the individuals and also demonstrate that the selected primers are highly informative and useful for further studies on *Moringa* genetic diversity and improvement programs in formation, conservation and utilization of third generation seed orchard. Using of R-ISSR markers have proven that genetic divergence is very high in *Moringa* genus and it can therefore be inferred from the data that the *M. peregrina* in Iran will be a very good germplasm material for the future breeding programs.

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