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Evaluation of Genetic Diversity of Iranian Wild *Alcea rosea* Population Using RAPD

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Abstract: The Hollyhock (*Alcea rose*) is a summer flowering biennial that is native to China and belongs to the Malvaceae family. Hollyhock is one of the most important medicinal plants. The first step for breeding purposes is to determine the genetic variation. There isn't any report on using molecular markers to evaluat genetic variation in Hollyhock *(Alcea rosea)*.There are various accessions of this plant in Iran; however, no comprehensive study to fully understand it has ever been carried out. In this study, Sixty accessions of Hollyhock native to Iran have been gathered from different parts of Iran to asses the genetic variability of the plant and to study their quantitative as well as qualitative features. we used RAPD technique to estimate the DNA polymorphism and genetic diversity among various accessions of this plant in Iran. Total of 108 primers were screened from which 18 primers were selected for RAPD analysis. 18 primers were used that produced 228 bands. Among them 219 (93.73%) were polymorphic and 9(6.27%) were bands monomorphic.Cluster analysis of the genotypes was performed using Jaccard's similarity coefficient and UPGMA method and as a result sixty accessions of Iranian Hollyhock were divided into 18 groups. The least and highest similarity coefficient were 0.31 and 0.75 respectively. Thus, these RAPD markers have the potential for identification of species/varieties and characterization of genetic variation within the accessions Hollyhock.

Key words: Alcea rosea · RAPD marker · Genetic diversity · Iran

INTRODUCTION

The Hollyhock (*Alcea rosea*) is a summer flowering biennial that is native to the China and belongs to the Malvaceae family [1]. Hollyhock (*Alcea rosea*) is a mainly south west Asian genus with about 70species. About 36 species has been found in Iran [2-3]. Depending on the cultivar, hollyhocks grow from two to nine feet tall. Hollyhocks are considered long-blooming (June to late August), with the flowers at the bottom of the raceme blooming first [1]. Hollyhock is one of the most important medicinal plants. The leaves and the roots were boiled and used to ease delivery and to increase milk production in nursing mothers [4]. The crushed leaves took away the itchiness of insect bites and relieved the discomfort of scalds and burns [4]. Malvaceous germplasm has been variously investigated by different molecular marker techniques but the earlier studies either focused on the comparison of the Malvaceae with other families in the order Malvales or to explore the genetic relationships and diversity within and among population and limited number of species in the same genus. Application of DNA marker technology as a tool for species identification has progressed rapidly during the last decade. Assessment of genetic diversity and detection of genetic distance in populations is important for breeding programs. Random amplified polymorphic DNA (RAPD) markers can be generated using short arbitrary primers to amplify genomic DNA, giving a genotype-specific pattern of bands. RAPD analysis should lead to the saturation of the genome without the requirement of previous genetic information [5]. RAPD is the most widely used molecular marker for analysis of DNA fingerprinting. The RAPD technique has become an increasingly popular tool in genetic studies. It is difficult to distinguish phenotypically similar cultivars using morphological and

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physiological methods or isozyme analyses [6]. The limitation of these analyses is that they are phenotypic based. Incontrast, DNA polymorphism offers direct observations of the plant genotype [7]. One of the techniques used for this purpose is Random Amplified Polymorphic DNA (RAPD) [8]. There isn't any report on using molecular markers for evaluating genetic variation in Hollyhock (*Alcea rosea)* but There are reports about the use of RAPD to discriminate intra- and inter-specific variation in Malvaceae family for example, Multani and Lyon [9] reported RAPD study in cotton in which they studies 14 Australian cultivars. Klips [10] Using isozymes for genetic affinity of the rare eastern Texas endemic Hibiscus dasycalyx (Malvaceae). Iqbal *et al.* [11] used the RAPD in the generation of DNA fingerprints of cotton varieties. They analyzed the RAPD markers to evaluate the genetic diversity of elite commercial varieties. In this study, we used RAPD technique to distinguish DNA polymorphism and genetic diversity various accessions of this plant in Iran.

MATERIALS AND METHODS

Plant Material: Sixty accessions of Hollyhock native to Iran have been gathered from different parts of Iran (Table 1). Seed all these accessions were grown in the same conditions in a greenhouse.

DNA Extraction and Rapd Amplifications: Seed accessions in a greenhouse were germinated and total genomic DNA was isolated from the very young of plants according to the protocol of Doyle and Doyle [12]. Genomic DNA was precipitated by isopropanol cold and finally washed with 76% ethanol and dissolved in 200 ml TE buffer. RNAase 3.5µl solution (10 mg/ml) treatment was given to remove RNA from the samples. Concentration and quality of each sample of DNA calculated from the Optical Density (OD) values at the wavelength 260 nm and 280 nm and DNA was regarded as being of good quality when, the ratio of OD_{260} to OD_{280} was near 1.9 and working solutions of genomic DNA (25 ng μl^{-1}) were prepared in sterile water. For PCR analysis, Eighteen 10-mer primers were used (Table 2). PCR was performed in a thermal cycler (Bio-Rad model i-Cycler)in a total volume of 25 µl containing 25 ng μl^{-1} of genomic DNA template, 1.25U Taq polymerase, 1.25 mM of each dNTP, 10 pmol random 10-mer primer, 1.5 Mm MgCl2,1X PCR buffer(10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl2) Amplifications were performed in a DNA Thermo-cycler(Bio-Rad model i- Cycler) programmed as follows: an initial denaturizing at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 2 min, followed by one final extension at 72°C for 4 min. Finally, the samples were either held at 4°C for direct use or stored at -20°C

Table 1: *Alcea Rosea* populations, their collection sites, codes, elevation, latitude, longitude and collection dates during the year 2009-2010.

Number	Population Name/Collection Site	Population Code	Elevation (m)	latitude East	Longitude North
1	Ilam A,B,C	1-3	1393	33.638	46.431
2	Khuzestan A,B,C	4-6	39	31.314	48.68
3	Kermanshah A,B,C	7-9	1389	34.314	47.065
4	Kurdistan A,B,C	10-12	1464	35.31	46.999
5	Bushehr A,B,C	13-15	9	28.974	50.834
6	Hamedan A,B,C	16-18	1824	34.795	48.514
7	Azarbaijan West A,B,C	19-21	1267	35.58	44.03
8	Golestan A,B,C	22-24	183	36.828	54.439
9	Gilan A,B,C	25-27	1	37.278	49.595
10	Mazandaran A,B,C	28-30	52	36.568	53.059
11	Azarbaijan East A,B,C	31-33	1395	38.08	46.292
12	Qazvin A,B,C	34-36	1290	36.262	50.017
13	Lorestan A,B,C	37-39	1147.8	33.26	45.17
14	Semnan A,B,C	40-42	1137	35.575	53.406
15	Tehran A,B,C	43-45	1149	35.672	51.424
16	Karaj A,B,C	46-48	1430	35.829	51.006
17	Chahar Mahal and Bakhtiari A,B,C	49-51	1800	31.09	49.28
18	Kohgiluyeh and Boyer Ahmad A,B,C	52-54	1989	30.717	51.567
19	Ardabil A,B,C	55-57	1333	38.249	48.301
20	Qom A,B,C	58-60	925	34.644	50.881

Table 2: Sequences of 18 primers and Polymorph bands and Polymorph rate of *Alcea Rosea*

Primer code	Nuclcotidc sequence	Total bands	Pic	Poly bands	Poly rate
J1	CCT GGG CTTA	17	0.55	17	100
R2	ACCGGGTTTC	12	0.91	11	91.66
R3	CTACCCGTGC	6	0.9	4	66.66
J2	GGGCACGCGA	13	0.97	13	100
J3	TGCTAGCCTC	14	0.88	14	100
R7	TCGGGATATG	17	0.85	17	100
R8	CACGGCGAGT	12	0.85	12	100
J12	CATGTGCTTG	13	0.62	13	100
J15	ATG ACGTTGA	6	0.8	4	66.66
JI5	ACATTGGGCG	15	0.21	15	100
A1	GTAATCGACG	16	0.87	12	100
A5	TTGAGACAGG	14	0.87	13	92.85
Z2	GGGCGAGTGC	13	0.88	13	100
Z9	ACAGCACCAT	9	0.83	8	88.88
BB01	ACACTGGCTG	9	0.82	8	88.88
BD04	ACCAGGTCAC	15	0.92	15	100
BD05	GTGCGGAGAG	12	0.81	11	91.66
BD17	GTTCGCTCCC	15	0.88	15	100
	Total	228bands	14.42	215bands	------
	Mean	12.66	0.8	11.94	93.73

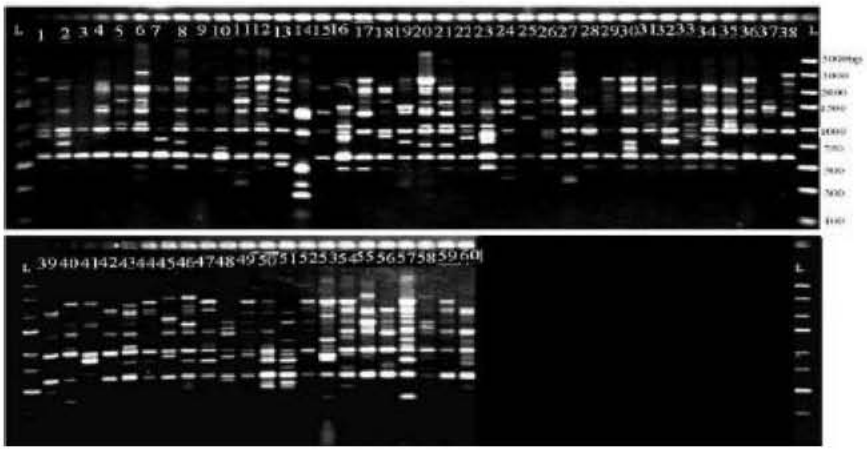

Fig. 1: Example of an agarose gel showing the amplified DNA patterns obtained with a RAPD-PCR reaction

until needed.After amplification PCR product was resolved by electrophoresis in 1/2% agarose gel for 2.16 h at 70 V with IX TBE buffer. Bands were visualized by staining with ethidium bromide (0.5 μg mL^{-1}) under UV light (Figure 1). The Band size was determined with the DNA size marker (SM1553 Fermentas).

Data Scoring and Analysis: For each genotype, the presence and absence of fragments were scored as 1 or 0, respectively. Similarity index was estimated using the jaccard's coefficient. dendrograms were drawn using NTSyS-pc Version 2.2 [13] softwares. Cluster analysis was performed using the unweighted pair-group method with an arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

Total of 108 primers (45primer of TibMolBiol and 63primer of NAPSE set) were screened from which 18 primers (4 primer of TibMolBiol and14 primer of NAPSE

set) were selected for RAPD analysis (Table 2). 18 primers were used that produced 228 bands. Among them 219(93.73%) were polymorphic and were 9(6.27%)bands monomorph.The highest number of productied bands were 17 (NAPSE set-J01 primer) and the highest polymorphic band ratio were 100% (J01, J02, J03, J12, R07, R08, J15, A1, BD04 and BD17 primers). The size of amplified bands ranged from approximately 300 bp to 3000 bp. Primers for each resolution power (Pic) in this experiment was calculated (Table 2). This factor represents the effectiveness of each indicator for the isolation and separation are studied samples.This index is calculated based on the following formula:

$Pic=1-\Sigma (Pi/Pn)^2$

Pi : Number of alleles per row.

Pn : Total number of alleles for each primer.

This value according to formula above for each of bands can be produced between one and zero is variable.Total resolving power (Pic) was 14.42 with the mean of 0.80. J02 primer showed most resolving power equal to 0.97. The lowest similarity (0.31) between golestan B, Ilam C and Boshehr C genotypes and highest (0.75) detected between Ilam A and Kermanshah C, Boshehr A, Ardabil C, Gilan C and Hamedan A (Figure 2). Cluster analysis based on jaccard's similarity coefficients and UPGMA method and at similarity level of 0.54, were divided the genotypes into 18 sub-clusters which Golestan B, Gilan A, Mazandaran B genotype was separated individually from others at distance of 0.33. Cluster analysis resulted in grouping of the 60 Hollyhock mass into seven main groups in 0.45 distance unit (Figure 2). Three genotype of the 60 accessions were included in first cluster, twenty-two accessions were in second cluster, three accessions in third cluster, five accessions in fourth cluster, twenty-seven accessions in Fifth cluster, two accessions in sixth cluster and three accessions in seventh cluster. There was little relationship between genetic divergence and geographical origins, so that the populations from similar geographical places (such as Tabriz a and Tabriz b) belonged to separate clusters. Conversely, populations from different geographical conditions (such as Ahvaze B and Tabriz B) relatively tended to be clustered in one subgroup of the dendrogram.These results show that there is high genetic diversity in Hollyhock. This high genetic diversity is good to the breeding to select excellent cultivars from the wild populations and can select new ones with high value and strong adaptation by crossing with different advantages and different habitation. The High diversity of Hollyhock is the reflection of adaptation to environment, which is beneficial to its propagation. Wild species represent the natural gene pool available for the genetic improvement of the cultivated Hollyhock. It will be worth to investigate specific traits in the wild species and they may be introgressed by sexual crossing or somatic hybridization into commercial varieties [14]. Generally, select parents with a higher general combining ability and long genetic distance can produce a hybrid with better yield performance [15-16]. But the identification of combining ability based on morphological characters is costly and time-consuming and may be influenced by environment factors. In contrast, molecular markers are not directly influenced by environmental effects or epistatic interactions and can provide large numbers of loci. These results show that RAPD is suitable for genetic diversity assessment in *Alcea rosea*. Classification of diversity in germplasm collections is important for both plant breeding and germplasm collection [17]. Genetic similarity values are always difficult to compare with other studies because genetic variability depends

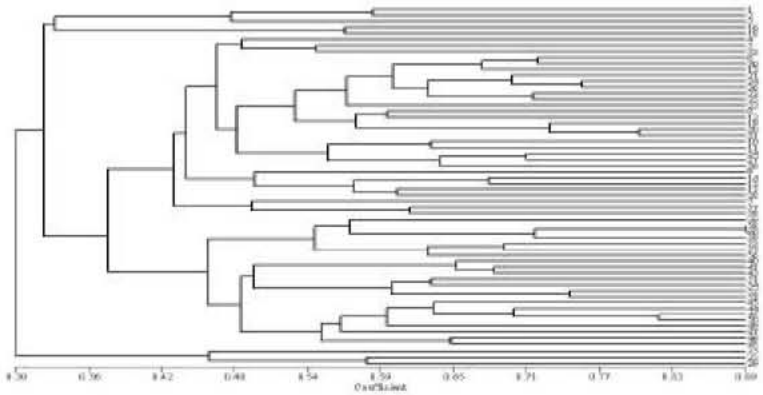

Fig. 2: Dendrogram obtained from RAPD data of 60 accessions of *Alcee Rosea* by UPGMA

heavily on factors including the history of the species, the reproductive system and ecology [18]. The RAPD analysis has been found to be a valuable DNA marker system to evaluate genetic diversity. The information about genetic similarity will be helpful to avoid any possibility of elite germplasm becoming genetically uniform. Efficiency and speed of plant breeding programs can be accelerated by (MAS) and permit persistent progress in the advancement of selected material [19] The information gathered here would be helpful in genomic mapping studies and for the development of Hollyhock accessions with wider and diverse genetic background to obtain improved plant productivity. These findings also proved that Iran is important center of Hollyhock diversity and Hollyhock germplasm of the Iran is very diverse. Wide variation in the desirable genotypes types in different regions substantiates the high level of genetic variability observed. Detection of genetic differences and discrimination of genetic relationship between Hollyhock species are for utilization of plant genetic resources.Our study clearly indicated that RAPD markers could be effectively used for genetic diversity studies among accessions of Hollyhock native to Iran.

CONCLUSION

Species-specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred. Genetically distinct cultivars were identified that could be potentially important sources of germplasm for Hollyhock improvement. The results obtained suggested that by using RAPD molecular markers the newly evolved Hollyhock can be easily differentiated from their parents. This would be a useful tool in identifying and protecting them from possible infringements in future.

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