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Random Amplified Polymorphic DNA profiling in detecting genetic variation in *Malva* L. species: edible and medicinal plants

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Abstract. *Malva* L. (mallow) is the genus within the Malvaceae Juss. family, which includes twenty- five-forty. species and several hybrids. This genus contains herbaceous annual, biennial, and perennial species that are native to regions of Africa, Asia, and Europe. *Malva* species contain a lot of mucilage, malvin, flavonoids, terpenoids, polysaccharides, and vitamin. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Malva* genetic diversity. Therefore, we collected and analyzed seven species from seven provinces of Iran regions. Overall, eighty-five plant specimens were collected. We showed significant differences in quantitative morphological characters in plant species. *Malva verticillata* L. depicted unbiased expected heterozygosity (UHe) in the range of 0.053. Shannon information was high (0.67) in *Malva parviflora* L. *Malva vericillata* showed the lowest value, 0.083. The observed number of alleles (*N*a) ranged from 1.16 to 2.33 in *Malva vericillata* and *Malva parviflora*. The effective number of alleles (*Ne*) was in the range of 1.078-1.922 for *Malva vericillata* and *Malva parviflora*.Gene flow (Nm) was relatively low (0.63) in *Malva*. The Mantel test showed correlation (r = 0.76, p=0.0001) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Malva* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Malva* species.Our aims were 1) to assess genetic diversity among *Malva* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa.

Keywords: population structure, gene flow, random amplified polymorphic DNA (RAPD), *Malva* species, network.

INTRODUCTION

The use of medicinal plants can be influenced by the economic condition, the high cost of medicines and the difficult access to public consultations. In addition to that, there is a difficulty of access by residents in rural areas to health care units located in urban areas. Moreover, the increase the trend for considering traditional knowledge that supports using natural

resources as an alternative to synthetic drugs (Battisti et al., 2013).

Malvaceae Juss. ('the mallows') is a botanical family with a rich diversity of species for textile, medicinal, and ornamental purposes. It consists of 4465 species and about 245 genera (Tate et al., 2005) and mallows present a cosmopolitan distribution, but with a high number of species in the tropics. The principle economic use of Malvaceae plants is as a source of natural fibers, the family providing perhaps the worlds three most important fiber crops plants of the family are also used for food, beverages, timber, in traditional medicine and in horticulture (la Duke and Dobley, 1995; Erbano et al. 2015; Frankham 2005; Ellegren and Galtier 2016; Turchetto et al. 2016). Many researches have been published on the ecology, taxonomy, genetic, cytology, chemotaxonomy, physiology, seed germination and economic uses of family Malvaceae such as (El-Rjoob and Omari 2009) in ecology; in taxonomy (Tate et al., 2005), in chemotaxonomy (Blunden et al., 2001; Gomez et al. 2005; Cires et al. 2013, Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d) and in genetic researches (Baum et al., 2004) studied the pollen.

The *Malva* genus has 25-40 species and it can be considered as an annual and/or biannual herb. Flowers with an epicalyx and 8-15 reticulated mericarps are the typical one (Fryxell, 1988; DellaGreca, et al., 2009). In medicine, mallow species are used in the treatment of respiratory, urinary, and digestive problems as they have high bactericidal, antiulcerogenic, anti-inflammatory, hepatoprotective, and antidiabetic activities (Pandey et al, 2012). The *Malva* genus is morphologically very diverse, but some species are hardly distinguishable based on morphological features (Escobar et al., 2009). Several studies have been conducted to clarify the taxonomic affiliation of *Malva* species using different features, such as molecular data (nuclear ribosomal DNA (rDNA), internal transcribed spacer (ITS) region, intron–exon splice junction (ISJ), and inter simple sequence repeat polymerase chain reaction (ISSR) markers) (Celka, et al., 2010), differentiation of seed and seed coat structure (El Naggar, 2001), morphology of pollen grains (El Naggar, 2004), epidermal structures and stem hairs (Akçin, and Özbucak, 2006), and plant morphological traits (Michael et al., 2009).

The variability in mallow species is due, at least in part, to hybridization. Natural crossings between *Malva pusilla* Sm. and *Malva neglecta* Wallr., *Malva alcea* L., and *Malva moschata* L. as well as *Malva sylvestris* L. and *Malva neglecta* were found in Europe. Ray (1995) stated that hybridization or polyploidy is probably a factor in the evolution of these species, but this aspect has not been investigated so far. The taxonomy and systematics of the *Malva* genus are still unclear and very complicated. Taxonomic doubts have appeared because of the high level of homoplasty in morphological traits that are usually used as diagnostic features (Escobar García, et al.,2009). Based on the flower structure, Dalby (1968) divided the *Malva* genus into two sections: *Bismalva* (with *Malva alcea, Malva excisa* Rchb., and *Malva moschata*) and *Malva* (*Malva neglecta, Malva pusilla, Malva sylvestris,* and *Malva verticillata*) A different classification based on ITS molecular markers as well as fruit morphology and seed structure was reported by Ray (1995), and two groups were distinguished: malvoid and lavateroid. A similar division was proposed by Escobar Garcia et al. (2009) based on five ITS molecular markers (matK plus trnK, ndhF, trnL-trnF, and psbA-trnH). These genetic relationships and the classification of *Malva* species were also confirmed by Celka et al. (2010) and Lo Bianco et al. (2017) based on ITS and ISSR molecular markers along with seed image analysis. Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (Esfandani-Bozchaloyi et al. 2017).

Taxonomical systematics studies were conducted in the past to identify the *Malva* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied seventy samples. Our aims were 1) to assess genetic diversity among *Malva* species 2) is there a correlation between species and geographical distance? 3) Genetic structure of populations and taxa 4) Are the *Malva* species able to exchange genes?

MATERIALS AND METHODS

Plant materials

Seven *Malva* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. Eighty-five plant samples (nine-fifteen per plant species) were examined for morphometry purposes (Figure 1). The random amplified polymorphic DNA analysis method was limited to eighty-five samples. We focused on the following species *Malva neglecta* Wallr., *Malva pusilla* Sm., *Malva*

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxa	Locality	Latitude	Longitude
Malva neglecta Wallr.	West Azerbaijan, Kaleybar	38°5'46.4604"	46°16'23"
Malva parviflora L.	Hormozgan, Bandar Abbas	27°33'12"	56°44'16"
Malva pusilla Sm.	Khuzestan, Behbahan	30°17'01"	50°54'10"
Malva sylvestris L.	Esfahan, Ardestan on road to Taleghan	32°15'44"	51°16'33"
Malva verticillata L.	Kerman, Hamun-e Jaz Murian	27°10'13"	58°33'19"
Malva nicaeensis All.	Mazandaran, 40 km Tonekabon to janat abad	35°10'16"	51°55'18"
Malva aegyptia L.	Golestan, Gorgan	35°13'19"	52°10'31"

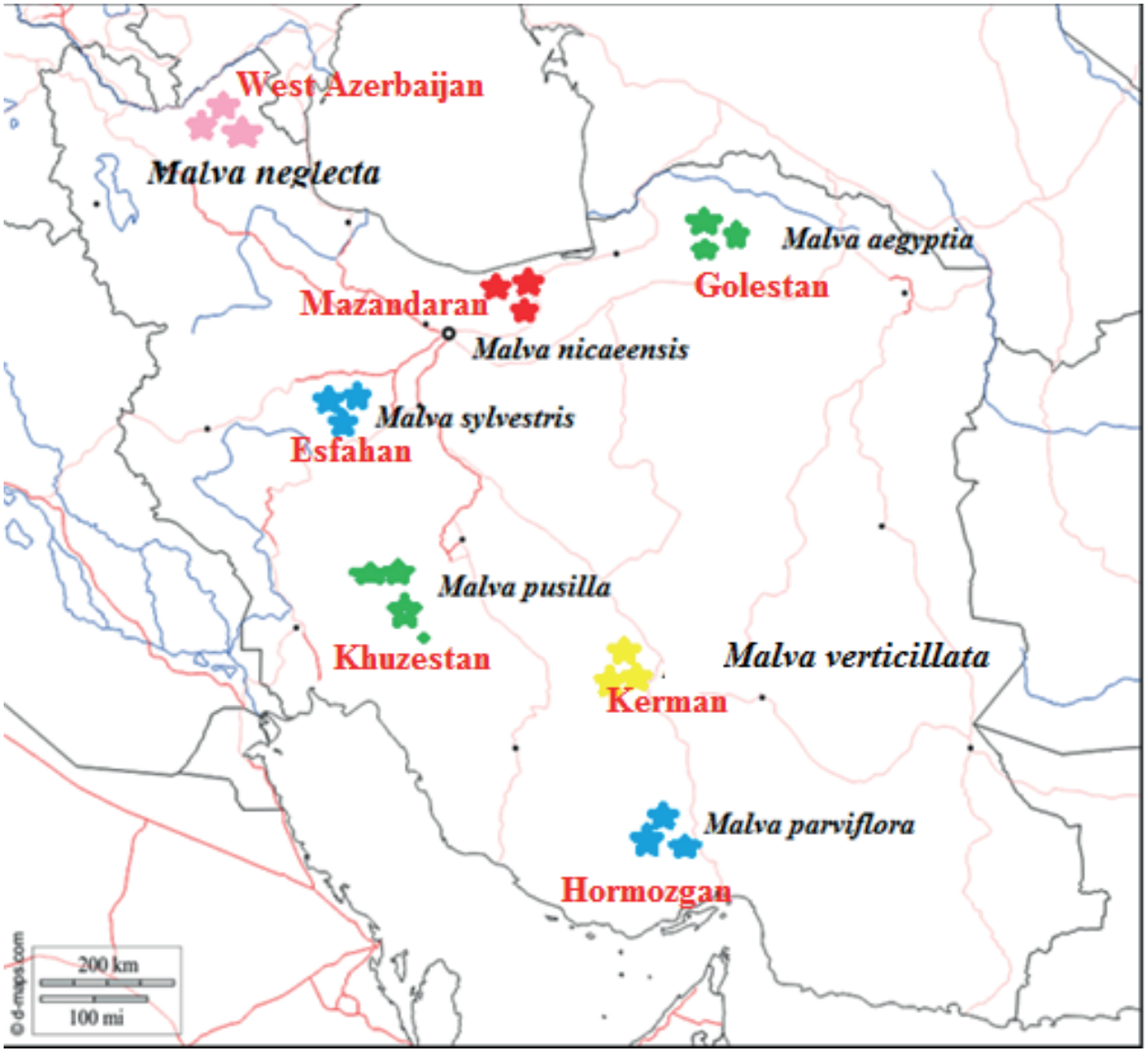

Figure 1. Presence of species in different regions of Iran.

sylvestris L., *Malva verticillata* L., *Malva nicaeensis* All., *Malva aegyptia* L. and *Malva parviflora* L. According to previous references, all the species were identified (Escobar García, et al., 2009; Ray, 1995).

Morphometry

In total thirty-eight morphological (ten qualitative, twenty-eight quantitative) characters were studied`. Five to ten plant specimens were randomly studied or morphological analyses**.** Data were transformed (Mean= 0, variance = 1) prior to ordination . Euclidean distance was implemented to cluster and ordinate plant species (Podani 2000).

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (Esfandani-Bozchaloyi et al. 2019). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD sets. We selected those primers (10) which could show clear bands and polymorphism (Table 2). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after this forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

We used an Unweighted pair group method with arithmetic mean (UPGMA) and Ward methods. Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (Podani 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (Podani 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism information content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (Ismail et al. 2019). Marker index was calculated according to the previous protocol (Heikrujam et al. 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diver-

Table 2. RAPD primers and other parameters. Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5'-AGGGGTCTTG-3'	13	12	92.31%	0.54	8.21	10.23	4.55
OPA-06	5'-GGTCCCTGAC-3'	17	17	100.00%	0.47	7.32	11.55	4.18
OPB-01	5'-GTTTCGCTCC-3'	11	9	96.89%	0.43	6.56	9.34	7.17
OPB-02	5'-TGATCCCTGG-3'	13	12	95.81%	0.34	4.21	6.60	5.59
OPC-04	5'-CCGCATCTAC-3'	12	12	100.00%	0.47	3.37	9.55	3.25
OPD-02	5'-GGACCCAACC-3'	11	11	100.00%	0.56	4.86	11.88	3.45
OPD-03	5'-GTCGCCGTCA-3'	9	7	84.99%	0.43	3.51	8.43	3.85
OPD-05	5'-TGAGCGGACA-3'	15	13	93.84%	0.66	4.66	11.33	4.67
OPD-08	5'-GTGTGCCCCA-3'	12	11	94.91%	0.48	5.21	12.50	5.65
OPD-11	5'-AGCGCCATTG-3'	14	13	95.74%	0.67	5.66	9.57	5.37
Mean		12.7	11.7	95.88%	0.55	5.5	9.4	4.8
Total		127	117					

sity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) (Shen et al. 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (Huson and Bryant 2006; Freeland et al. 2011). The Mantel test was carried out to find the correlation between genetic and geographical distances (Podani 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (Peakall and Smouse 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in PopGene ver. 1.32 (Yeh et al. 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (Evanno et al. 2005). First data were scored as dominant markers (ISSR) so we used from STRUCTURE analysis for estimate the parameters that related to gene flow among studied population. Burn-in = 10000, and 10 runs were performed for relationship between Genetic structure and distance of geographical. Maximum likelihood method and Bayesian Information Criterion (BIC) was studied by structure analysis (Falush *et al.* 2007; Evanno *et al.* 2005; Meirmans 2012).

RESULTS

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Principal component results explained 67% variation. First component of PCA demonstrated 49% of the total variation. Leaf morphology and traits such as calyx length, calyx width positively correlated with corolla length, corolla color (>0.7). The second and third components explained floral characters such as corolla apex, seed length and number of segment stem leaves. Unweighted pair group method with arithmetic mean (UPGMA) and principal coordinate analysis (PCoA) plots showed symmetrical results (Figure 2, Figure 3). Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Morphological characters divided *Malva* species into two groups, as evident in the UPGMA tree

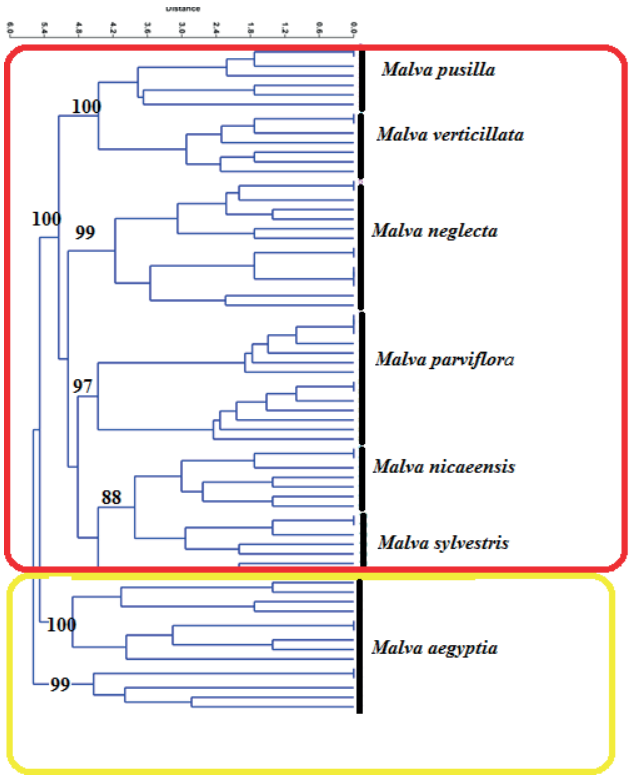

Figure 2. UPGMA clusters of morphological characters revealing species delimitation in *Malva* species.

(Figure 2). Populations belonging to *Malva aegyptia* were in the first group. On the other hand, the second group consisted of two sub-groups. *Malva pusilla* and *Malva verticillata* formed the first sub-group. *Malva neglecta, Malva sylvestris, Malva parviflora, Malva nicaeensis* formed the second sub-group. These groups and subgroups were formed due to morphological differences among the individuals of *Malva*. Our PCoA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 3). Identical results were also reported in the UPGMA tree (Figure 2).

Species identification and genetic diversity

The primers, i.e., OPC-04, OPB-01, OPA-05 and OPD-11 could amplify plant (*Malva* species) DNA (Figure 4). 119 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPA-06. OPD-03 had the lowest polymorphic bands. The average polymorphic bands ranged to 11.9 for each primer. The polymorphic information content (PIC) had

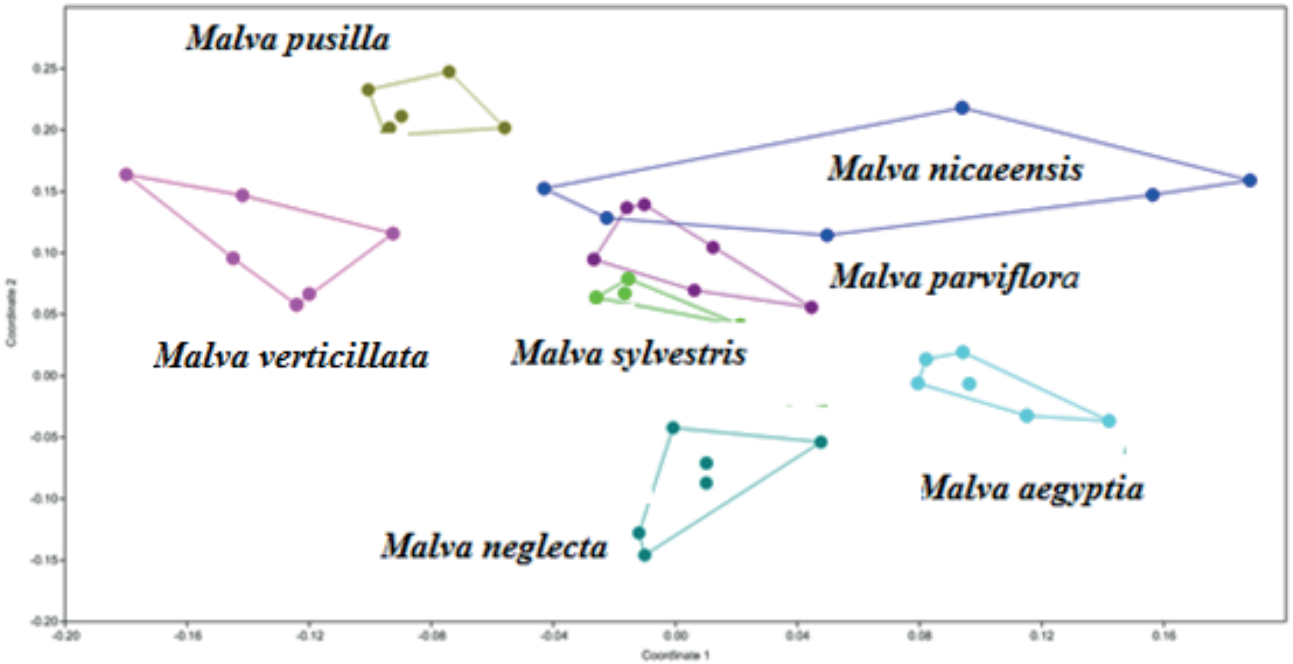

Figure 3. PCoA plot morphological characters revealing species delimitation in *Malva* species.

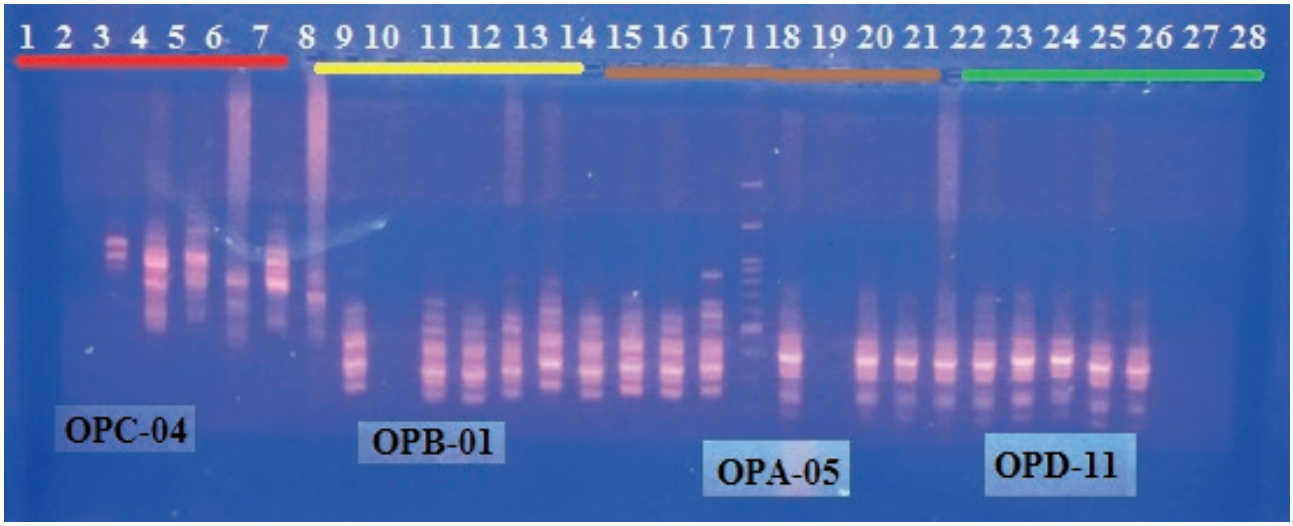

Figure 4. Gel Electrophoresis image of DNA fragments of *Malva* species. L = Ladder 100 bp. Arrows show polymorphic bands.1,8,15,22: *Malva neglecta* 2,9,16,23: *Malva parviflora* 3,10,17,24: *Malva pusilla*. 4,11,18,25: *Malva sylvestris* 5,12,19,26: *Malva verticillata* 6,13,20,27: *Malva nicaeensis*7,14,21,28: *Malva aegyptia.*

values in the range of 0.34 (OPB-02) to 0.67 (OPD- 011). Primers had 0.55 average polymorphic information content values.

Marker index (MI) values were 3.25 (OPC-04) to 7.17 (OPB-01), with an average of 4.8 per primer. Effective multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 6.60 (OPB-02) to 12.50 (OPD-08) EMR values. EMR values averaged 9.4 per primer (Table 2). All the necessary genetic features calculated of seven *Malva* species are shown (Table 3). *Malva verticillata* depicted unbiased expected heterozygosity (UHe) in the range of 0.053. Shannon information was high (0.67) in *Malva parviflora*. *Malva verticillata* showed the lowest value, 0.083. The observed

Table 3. Genetic diversity variables of *Malva* (N = number of samples, Na= number of different alleles, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism in populations).

Taxon	N	Na	Ne	I	He	UHe	%P
Malva neglecta	10.000	1.500	1.311	0.279	0.267	0.187	50.00%
Malva parviflora	9.000	2.333	1.922	0.670	0.333	0.417	83.33%
Malva pusilla	12.000	1.500	1.441	0.330	0.233	0.233	50.00%
Malva sylvestris	13.000	1.333	1.232	0.196	0.200	0.133	33.33%
Malva verticillata	10.000	1.167	1.078	0.083	0.150	0.053	16.67%
Malva nicaeensis	15.000	1.200	1.462	0.337	0.290	0.240	50.00%
Malva aegyptia	15.000	1.433	1.196	0.150	0.183	0.090	19.67%

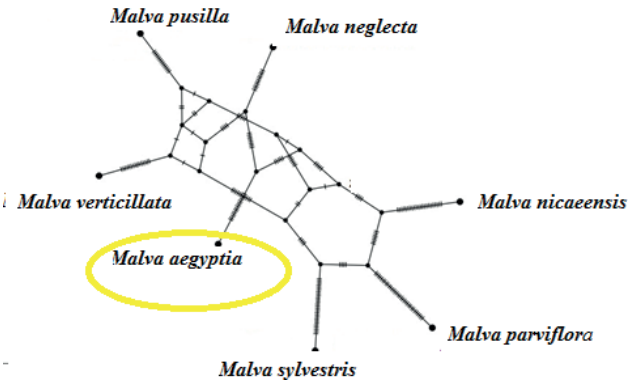

Figure 5. Integer NJ net tree produced while using RAPD data.

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	Df	SS	MS	Est. Var	%
Among Regions	5	42.297	12.648	0.337	20%
Among Pops	15	96.827	8.802	0.774	50%
Among Indiv	59	64.383	2.130	0.363	18%
Within Indiv	65	14.500	0.204	0.204	12%
Total	133	215.007		1.678	100%

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance.

Table 5. The Nei genetic similarity (*Gs*) estimates using RAPD markers.

pop1	pop2	pop3	pop4	pop5	pop6	pop7	
1.000							pop1
0.766	1.000						pop2
0.760	0.764	1.000					pop3
0.750	0.730	0.827	1.000				pop4
0.774	0.797	0.762	0.794	1.000			pop5
0.733	0.770	0.727	0.707	0.856	1.000		pop6
0.679	0.722	0.750	0.704	0.719	0.698	1.000	pop7

number of alleles (*Na*) ranged from 1.16 to 2.33 in *Malva verticillata* and *Malva parviflora*. The effective number of alleles (*Ne*) was in the range of 1.078-1.922 for *Malva veritcillata* and *Malva parviflora*. Gene flow (Nm) was relatively low (0.63) in *Malva*.

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Malva* species (P = 0.001). AMOVA showed that 50% of genetic variation was among the species. Relative less variation (12%) was reported within the species (Table 4). Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.567, P = 0.001) and D_est values (0.876, p = 0.001).

The neighbor-joining tree and MDS plot of *Malva* populations based on RAPD data produced similar results therefore only neighbor-joining tree is presented and discussed (Fig. 5). NJ net tree revealed that the seven species are well differentiated on the genetic grounds. In both UPGMA and NJ trees, samples of the *Malva aegyptia* were placed far from each other. *Malva pusilla* was placed close to *Malva verticillata* , and far from *Malva aegyptia* . In both analyses, *Malva nicaeensis* showed closer affinity with *Malva sylvestris, Malva parviflora.* Genetic distance of the two subsp. was estimated to be 1.66 by Kimura 2p distance.

Gene flow (Nm) was relatively low (0.63) in *Malva* species. Genetic identity and phylogenetic distance in the *Rindera* members are mentioned (Table 5). *Malva verticillata* and *Malva nicaeensis* were genetically closely related (0.856) to each other. *Malva nicaeensis* and *Malva aegyptia* were dissimilar due to low (0.694) genetic similarity. The mantel test showed correlation (r = 0.76, p=0.0001) between genetic and geographical distances.

The Evanno test showed ΔK =6 (Figure 6). Figure 6, showed the genetic details of the *Malva* species. According to STRUCTURE analysis *Malva pusilla* and *Malva aegyptia* were closely related to common alleles (Figure 6). The rest of the *Malva* species are genetically differentiated due to different allelic structures (Figure 6). The neighbor-joining plot also showed the same result. Limited gene flow results were supported by K-Means and STRUCTURE analyses too. We could not identify substantial gene flow among the *Malva* species. This result is in agreement with grouping we obtained with Neighbor- joining (Figure 5), as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise

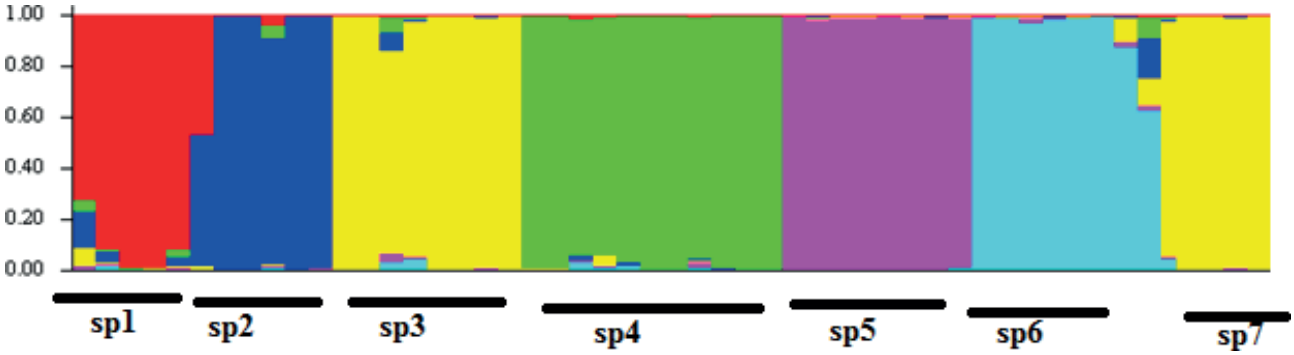

Figure 6. STRUCTURE plot of RAPD data in *Malva* populations **s**tudied. 1. *Malva neglecta;* 2. *Malva parviflora;* 3. *Malva pusilla*; 4. *Malva sylvestris;* 5. *Malva verticillata;* 6. *Malva nicaeensis;* 7. *Malva aegyptia.*

very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Malva* populations.

DISCUSSION

The *Malva* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Malva* species (Ray 1995; Escobar García, et al., 2009). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (Erbano et al. 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (Erbano et al. 2015; Abeshu & Zewdu 2020.; Amar et al 2021; Beltran et al. 2021). We examined genetic diversity in *Malva* by morphological and molecular methods (Das et al 2021; Gutierrez-Pacheco et al 2021; Hindersah et al 2021; Jordaan & Rooyen et al 2021). We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Malva*. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Malva* species. PCoA plot results also confirmed the application of morphological characters to separate *Malva* species. The present study also highlighted that morphological characters such as corolla color, leaf shape, leaf length, stamens position, leaf margin and corolla lenght could delimit the *Malva* group. The *Malva* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits.

In our study, morphology and genetic diversity in seven taxa of *Malva* species are given in detail for the first time. The aim of the present study was to find diagnostic features to separate species of *Malva* in Iran. Morphological characters are considered as an useful tool for the identification of the species, as indicated previously Ray (1995).

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. Very little attention has been given to the analysis at interspecific and intergeneric levels. La Duke and Dobley (1995) has the only worth mentioning work in this regard. Their results showed that, the genetic relationships and diversity within and between 12 malvaceous species belonging to five genera are investigated by using the Amplified fragment length polymorphism (AFLP).

Shaheen et al., (2009) with used AFLP (Amplified fragment length polymorphism) marker to explore phenetic relationships and diversity within and between 13 Malvaceae species belonging to 5 different genera. Their primary objective of the study was to evaluate the taxonomic potential, usefulness and applicability of AFLP marker system to reconstruct genetic relationships at interspecific and intergeneric level in Malvaceae. Two primer pairs produced a total of 73 bands, of which 70 were polymorphic.

According to Celka et al (2010) two categories of DNA markers were used to determine genetic relationships among eight *Malva* taxa. A maximum parsimony analysis validated the division of the genus *Malva* into the sections *Bismalva* and *Malva*. The species classified into those sections formed separate clusters. *Malva moschata* was a distinctive species in the section *Bismalva*, as confirmed by previous genetic research based on ITS and cpDNA sequence analyses. The applied markers

revealed a very high level of genetic identity between *Malva alcea* and *Malva excisa* and enabled molecular identification of *M. alcea* var. *fastigiata*.

Jedrzejczyk and Rewers (2020) applied flow cytometry and inter simple sequence repeat polymerase chain reaction (ISSR-PCR) for fast and accurate species identification. Genome size estimation by flow cytometry was proposed as the first-choice method for quick accession screening. Out of the 12 tested accessions, it was possible to identify six genotypes based on genome size estimation, whereas all species and varieties were identified using ISSR markers. Flow cytometric analyses revealed that *Malva* species possessed very small (1.45–2.77 pg/2C), small (2.81–3.80 pg/2C), and intermediate (11.06 pg/2C) genomes, but the majority of accessions possessed very small genomes. The relationships between the investigated accessions showed the presence of two clusters representing malvoid and lavateroid group of species. Their results showed that Flow cytometry and ISSR molecular markers can be effectively used in the identification and genetic characterization of *Malva* species.

Until now, molecular studies using ISSR markers conducted in the *Malva* genus have only included a few species (Celka, et al., 2012). All primers used in ISSR-PCRs for the *Malva* genus revealed 100% polymorphism between all accessions. Therefore, it was possible to identify all tested species. Moreover, for *Malva verticillata* taxon, it was possible to distinguish all studied varieties. The usefulness of most of the used ISSR primers was also confirmed in *Ocimum* L., *Origanum* L. and *Mentha* L. identification (Lo Bianco, et al., 2017). The systematics of the *Malva* genus and closely related genera is complicated. Moreover, the relationships obtained from molecular studies do not confirm traditional classification (Escobar García, et al., 2009). So far, only molecular analysis relying on rDNA ITS sequences and ISSR markers have shed light on taxonomical relationships between *Malva* species (Escobar García, et al., 2009). Phylogenetic analyses of rDNA ITS sequences indicated the presence of two well-supported clusters within the mallow species (malvoid and lavateroid clades), which is consistent with the presented data.

Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Malva* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Malva* population. We also reported high genetic diversity, which clearly shows the *Malva* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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