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Comparative study and genetic diversity in *Malva* using srap molecular markers

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Abstract. The *Malva* genus has 25-40 species and it can be considered as an annual and/or biannual herb. *Malva* species are indicated with potential therapeutic as cicatrizing and analgesic by the Ministry of Health. The aim of this study was to analyze SRAP (Sequence-related amplified polymorphism) markers in a total of 70 accessions of *Malva* species, which included five species *Malva neglecta* Wallr., *Malva pusilla* Sm., *Malva sylvestris* L., *Malva verticillata* L., *Malva nicaeensis* All.. A total of 89 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction amplifications (PCR) amplification of five *Malva* species. These bands were produced with the combinations of 5 selective primers. The total number of amplified fragments ranged from 10 to 27. The predicted unbiased gene diversity (UHe) varied between 0.077 (*Malva sylvestris*) and 0.382 (*Malva pusilla*). The genetic similarities between three species are estimated from 0.70 to 0.91. Neighbor-Joining tree results showed two major clusters. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *Malva pusilla* and *Malva aegyptia* had the lowest similarity. Our results provided great molecular identification of all assayed genotypes, which have shown that there is large quantity of genetic diversity among the *Malva* accessions. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using NJ approaches. Current results have implications in breeding and conservation programs.

Keywords: Sequence-related amplified polymorphism, Genetic Diversity, Medicinal Plants *Malva,*Taxonomy.

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 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). Given the significance of genetic diversity in conservation strategies, it is of utmost importance to disentangle genetic diversity in plant species, particularly threatened and rare species (Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d).

The indiscriminate use of plants due to the lack of phytochemical, pharmacological and mainly toxicological knowledge is of great concern for public health. The correct identification of medicinal plant species is necessary, especially when they are processed in order to avoid misuse of medicinal plants (Romitelli & Martins, 2013). The *Malva* genus presents different species with therapeutic potential and inadequate consumption can occur due to the incorrect identification of the plant in the market.

Malvaceae or the mallow family is the family of flowering plants containing over 200 genera with close to 2300 species (la Duke and Dobley 1995). 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 in chemotaxonomy (Blunden et al., 2001) and in genetic researches (Baum et al., 2004) studied the pollen.

Malva L. (mallow) is the genus within the Malvaceae Juss. family, which includes 25–40 species and several hybrids (Ray 1995). This genus contains herbaceous annual, biennial, and perennial species that are native to regions of Africa, Asia, and Europe (Shaheen 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), 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 *M. pusilla* Sm. and *M. neglecta*, *M. alcea* L., and *M. moschata* L. as well as *M. sylvestris* and *M. 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 (Chen et al. 2021 ; BI et al. 2021). Sequence-related amplified polymorphism (SRAP) is PCR –based marker system. It is one of the efficient and simple marker systems to study gene mapping and gene tagging in plant species (Li and Quiros 2001), and SRAP are potential markers to assess plant systematics and genetic diversity studies (Robarts and Wolfe 2014). Previously, Wu et al. (2010) assessed genetic diversity and population structure in *Pogostemon cablin* with the aid of SRAP markers. SRAP markers were successfully implemented in Lamiaceae, Geraniacea, Caryophyllacea and Rosaceae family to study natural populations and variations within the family (Peng et al., 2021; Ma et al.,2021). Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using NJ approaches. Current results have implications in breeding and conservation programs. The present study is the first report on genetic diversity and phylogenetic relationships between and within *Malva* species in Iraq using SRAP markers.

MATERIALS AND METHODS

Plants collection

Five wild *Malva* species (*Malva neglecta* Wallr., *Malva pusilla* Sm., *Malva sylvestris* L., *Malva vericillata* L., *Malva nicaeensis* All..) in Halabja, Sulaimanieh, Kalar, Chamchamal and Basreh Provinces of Iraq were selected and sampled during July-August 2015-2020. Morphometric and SRAP analyses on 70 plant accessions were carried out. Five to twelve samples from each population belonging to three different species were selected based on other eco-geographic characteristics. Detailed information about locations of samples and geographical distribution of species are mentioned.

Morphological studies

Five to twelve samples from each species were used for Morphometry. In total 36 morphological (13 qualitative, 23 quantitative) characters were studied. Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000). Morphological characters studied are: corolla shape, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length, corolla length, corolla width, corolla apex, leaf length and leaf width, leaf apex, leaf margins, leaf shape, leaf gland and bract margins.

Sequence-related amplified polymorphism method

Fresh leaves were used randomly from one to twelve plants. These were dried with silica gel powder. Genomic DNA was extracted while following previous protocol. SRAP assay was performed as described previously (Li and Quiros 2001). Five SRAP in different primer combinations were used (Table 1). The overall reaction volume consisted of 25 µl. This PCR reaction was carried out in Techne thermocycler (Germany). The following cycles and programs were observed. The initial denaturation step was performed for 5 minutes at 94°C. The initial denaturation step was followed by 40 cycles for 1 minute at 94°C; 1 minute at 52-57°C, and 2 minutes at 72°C. The reaction was completed by a final extension step of 7-10 min at 72°C. Staining was performed with the aid of ethidium bromide. DNA bands/fragments were compared against a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

ANOVA (Analysis of variance) was conducted to assess morphological differences among species. Principal component analysis (PCA) was implemented to identify variable morphological characters in *Malva* species. Multivariate statistical analyses i.e., PC analysis, were performed in PAST software version 2.17 (Hammer et al. 2001).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were scored present (1) and absent (0), respectively. Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Furthermore, the polymorphic ratio was assessed based on NPL/NTL values. Polymorphism information content was calculated as previously suggested by Roldan-Ruiz et al. (2000). Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined. Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking. Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. To assess the population structure of the pistachio genotypes, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE () was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard et al. 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno et al. (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k. Pairwise genetic similarity between species was evaluated to reveal genetic affinity between species (Jaccard, 1908). Unbiased expected heterozygosity and Shannon information index were calculated in GenAlEx 6.4 software (Peakall and Smouse, 2006).

Table 1. SRAP primer information and results.

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em3-Me4	**27**	27	100.00%	0.55	33.24
Em3-Me1	16	10	75.00%	0.11	55.55
Em4-Me1	17	17	100.00%	0.39	11.23
Em5-Me1	10	10	100.00%	0.50	38.55
Em5-Me2	19	13	66.00%	0.32	44.65
Mean	19	15	83.10%	0.44	39.23
Total	89	80			

RESULTS

Morphometry

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 60% cumulative variation. The first PCA axis explained 40% of the total variation. The highest correlation (> 0.7) was shown by morphological characters such as corolla apex, seed length; number of segment stem leaves; calyx length, calyx width; bract length and leaf shape. The morphological characters of five *Malva* species are shown in PCA plot (Figure 1). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Malva* species and separated each groups.

Species identification and genetic diversity

Five (5) suitable primer combinations (PCs), out of 25 PCs were screened in this research. Figure 2 illustrates the banding pattern of Em2-Me4 and Em4-Me1 primer by the SRAP marker profile. Eighty (80) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 100bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 27 and 10 for Em3-Me4 and Em5-Me1, respectively. Each primer produced 15 polymorphic bands on average. The PIC ranged from 0.11 (Em3-Me1) to 0.55 (Em1-Me4) for the 5 SRAP primers, with an average of 0.44 per primer. RP of the primers ranged from 11.23 (Em4-Me1) to 55.55 (Em3-Me1) with an average of 39.23 per primer (Table 2). The calculated genetic parameters of *Malva* species are shown (Table 2). The unbiased heterozygosity (H) varied between 0.077 (*Malva sylvestris*) and 0.382 (*Malva pusilla*) with a mean of 0.23. Shannon's information index (I) was maximum in *Malva nicaeensis* (0.395), where as we recorded minimum Shannon's information index in *Malva sylvestris* (0.13). The observed number of alleles (*Na*) ranged from 1.11 in *Malva aegyptia* to 1.580 in *Malva nicaeensis*. The significant number of alleles (*Ne*) ranged from 1.077 (*Malva nicaeensis*) to 1.432 (*Malva pusilla*).

Table 2. Genetic diversity parameters in the different *Malva* populations, species, and cultivars; Abbreviations.

Population	%P	N	Na	Ne	I	He	UHe
Malva pusilla Sm.	53.00%	15.000	1.500	1.432	0.388	0.310	0.382
Malva sylvestris L.	22.11%	10.000	1.333	1.177	0.130	0.033	0.077
Malva vericillata L.	33.33%	12.000	1.300	1.388	0.271	0.167	0.288
Malva nicaeensis All.	49.00%	17.000	1.580	1.077	0.395	0.156	0.277
Malva aegyptia L.	30.33%	13.000	1.110	1.366	0.299	0.238	0.144

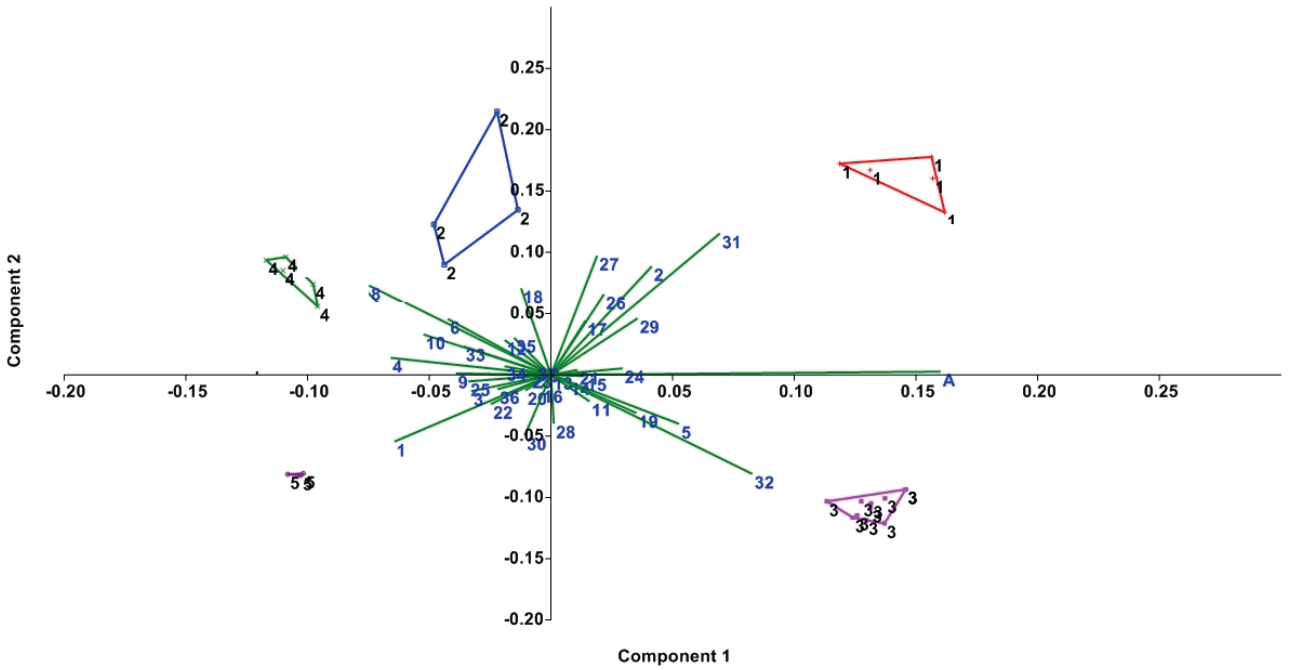

Figure 1. Morphological characters analysis of the *Malva* species by PCoA plot.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	70	2701.394	77.782	10.166	66%	66%
Within Pops	10	111.449	390.19	27.833	34%	
Total	80	2875.807		37.060	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; ***Φ***PT: proportion of the total genetic variance among individuals within an accession, ($P < 0.001$).

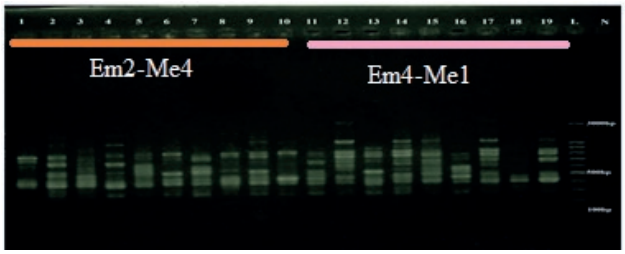

Figure 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1, 7: *Malva neglecta* 2,8: *Malva parviflora* 3,9: *Malva pusilla*. 4,10: *Malva sylvestris* 5,11: *Malva vericillata* 6,12: *Malva nicaeensis* = Ladder 100 bp.

Analysis of Molecular Variance results in significant genetic difference (p = 0.01) among *Malva* species. The majority of genetic variation occurred among species. AMOVA findings revealed that 66% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 3). Genetic difference between *Malva* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.578, p = 0.01) and D_est values (0.829, p = 0.01) .

NJ tree and UPGMA clustering produced similar results therefore only NJ tree is presented and discussed (Figure 3). This result show that molecular characters studied can delimit *Malva* species in two different major clusters or groups. In general, two major clusters were formed in NJ tree (Fig. 3), 20 individual of *Malva nicaeensis* and *Malva aegyptia* formed a single cluster. Cluster II contained two sub-clusters, and most of individual *Malva pusilla*; *Malva sylvestris* and *Malva vericillata* formed cluster II. There were 50 individuals in this cluster.

We detected strong correlation between geographical and genetic distances (r = 0.45, p=0.0002) and gene flow ($N_{m)}$ score of 0.48 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Table not included). The findings suggested that there was the highest degree of genetic similarity (0.91) between *Malva vericillata* and *Malva nicaeensis*. On the contrary to this, *Malva pusilla* and *Malva aegyptia* (0.70) had lowest genetic resemblance.

The Evanno test ΔK =5 (Figure not included), showed the genetic details of the *Malva* species. According to STRUCTURE analysis, the *Malva* species are genetically differentiated due to different allelic structures (Figure not included). 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 NJ tree (Figure 3), as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise 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.

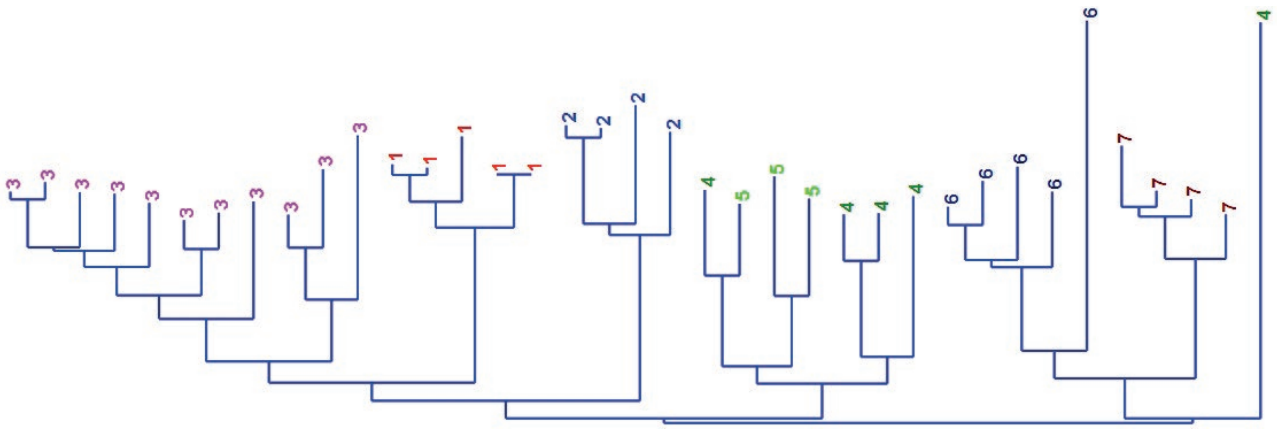

Figure 3. Neighbor-Joining tree of populations in *Malva* species based on SRAP molecular markers.

DISCUSSION

In the present study, we used morphological and molecular (SRAP) data to evaluate species relationships in *Malva*. Morphological analyses of *Malva* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated from each other. PCA analysis suggests that morphological characters such as corolla shape, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length have the potentials to identify and delimitate *Malva* species. Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Malva* species. Morphological characters including corolla length, corolla width, corolla apex, leaf length and leaf width, leaf apex, leaf margins, leaf shape, leaf gland and bract margins play key role in plant systematics and taxonomy. Our work also highlighted the significance of morphological characters and molecular data to identify and study species genetic diversity. In general, genetic relationships obtained from SRAP data coincides with morphometric results. This is in accordance with the parameters of AMOVA and genetic diversity results. SRAP molecular markers detected clear genetic difference among species. These results indicate that SRAP have potentials to study plant systematics and taxonomy in *Malva* members.

Given the negative impact of biodiversity threats and overexploitation of *Malva* plant species in Iran, it is necessary to conduct genetic diversity studies on *Malva* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Jia *et al.* 2020; Shi *et al*., 2021; Zheng *et al.,* 2021; Zhu *et al*., 2021). Genetic diversity studies are conducted through appropriate selection of primers and indexes including Polymorphic information content (PIC) and marker index (MI) are important indexes to fathom genetic variation in species (Wang *et al*., 2021; Yin *et al*., 2021; Zhao *et al*., 2021). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Sivaprakash et al. 2004).

In the present work, 5 *Malva* species were characterized with 5 SRAP markers. The results confirm the efficiency of microsatellite markers for fingerprinting purposes. Our results demonstrated that the PIC ranged from 0.11 (Em3-Me1) to 0.55 (Em3-Me4) for the 5 SRAP primers, with an average of 0.44 per primer. RP of the primers ranged from 11.23 (Em4-Me1) to 55.55 (Em3-Me1) with an average of 39.23 per primer.

Diversity study in Malva species

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. *M. 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 *M. alcea* and *M. 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 clus-

ters 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.

CONCLUSIONS

The present study investigated the molecular variation of five species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Malva* species. This was first attempt to assess genetic diversity through Sequence-related amplified polymorphism and morphometrics analysis in Iraq. Current study reported two major clusters. These two major groups were separated on the basis of genetic and morphological characters. The genetic similarities between three species was estimated from 0.70 to 0.91. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism envloved in the ecology of *Malva* species . Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinitiy among *Malva* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iraq.

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