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GENETIC DIVERSITY AND RELATIONSHIPS AMONG MEDICINAL SPECIES OF *MALVA* L. (MALVACEAE) BASED ON ISSR MARKERS

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Abstract

The genus *Malva* L., popularly known as mallow, grows spontaneously in almost all of Europe and the Mediterranean region. This genus is morphologically very diverse and its species are used in the treatment of respiratory, urinary, and digestive problems, but some species are hardly distinguishable based on morphological features. We, therefore, performed a molecular data analysis for this genus. Amplification of genomic DNA of 90 randomly selected samples of six species of *Malva* using 5 primers produced 83 bands, of which 77 were found to be polymorphic (90.99%). The high average PIC and MI values revealed the high capacity of ISSR primers to detect polymorphic loci among *Malva* species. The genetic similarities of the six collections were estimated from 0.73 to 0.90. ISSR analysis revealed that *Malva parviflora* and *M. vericillata* had the lowest similarity whereas, *M. neglecta* and *M. sylvestris* had the highest similarity.

Introduction

Identifying the accurate boundaries of a species is critical to have a better perspective of any biological studies. Therefore, species delimitation is a subject of an extensive part of studies in the framework of biology. However, defining the criterion which could address the boundaries of species is a matter of debate. Wild relatives of crops contain genes with great potential for use in breeding programs and constitute a part of their gene pool. In addition, the study of intra-specific levels of genetic variation and investigation of the genetic structure of wild populations is crucial for the development of effective conservation strategies.

Malvaceae ('the mallows') is a family with a rich diversity of species for textile, medicinal, and ornamental purposes. It consists of 2300 species and about 200 genera and mallows present a cosmopolitan distribution, but with a high number of species in the tropics (Ray, 1995).

The genus *Malva* L., popularly known as mallow, grows spontaneously in almost all of Europe and the Mediterranean region. It 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 ones. Ray (1995) and Escobar *et al*. (2009) relate their similarity to the *Lavatera* L. genus, where the bracteoles of the epicalyces are joined at the base, in contrast to in *Malva* where they are totally separated. Although there is inconsistency for some species in relation to the fusion of bracts and other characteristics. Molecular studies have also shown that the separation of these two genera based on this morphology is artificial and unsatisfactory (Escobar *et al*., 2009).

Malva species are potential therapeutic as cicatrizing and analgesic, considered by the Ministry of Health. Within the genus, some species (e.g., *M. parviflora*, *M. pusilla* Sm., *M. nicaeensis*, and *M. neglecta*) are regularly misidentified based on morphological features (Della Greca *et al*., 2009). The phylogenetic relationships and taxonomic organization of the *Malva* genus are still unclear. Therefore, molecular analysis can be useful in species identification and the

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study of the genetic relationships between *Malva* taxa (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), the 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* 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 (Escobar García *et al.*, 2009). Based on the flower structure, Dalby (1968) divided the *Malva* genus into two sections: *Bismalva* (with *M. alcea, M. excisa* Rchb., and *M. moschata*) and *Malva* (*M. neglecta, M. pusilla, M. sylvestris*, and *M. 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 *viz*., malvoid and lavateroid were distinguished. A similar division was proposed by Escobar *et al*. (2009) based on five ITS molecular markers (*mat*K, *trn*K, *ndh*F, *trn*L-*trn*F, and *psb*A-*trn*H). 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. Most of the *Malva* species are polyploids with the base chromosome number $n = x = 7$ (3,43). Numerous species are hexaploids, where the chromosome number is in the range of 40 to 44, and a few species possess higher numbers of chromosomes.

Molecular markers provide a powerful tool for studying genetic diversity. Among advanced genetic markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers have been widely used for diversity analyses. RAPD technique is quick, easy and requires no prior sequence information. The technique detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence. ISSR marker involves PCR amplification of DNA by a single 16-18 bp. long primer composed of a repeated sequence anchored at the 3' or 5' end of 2-4 arbitrary nucleotides. The technique is rapid, simple, inexpensive and more reproducible than RAPD.

The present investigation has been carried out to evaluate the genetic diversity and relationships among different *Malva* species using new gene-targeted molecular markers, i.e., ISSR markers. This is the first study on the use of ISSR markers in *Malva* genus in Iraq. In this study we performed a molecular study of 90 collected specimens of six *Malva* species. We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Malva* species in Iraq?

Materials and Methods

Plant materials

A total of 90 individuals were sampled representing six geographical populations belonging to six *Malva* species of Iraq during July-Agust 2017-2020. For ISSR analysis we used 90 plant

accessions (Five to twelve samples from each populations) belonging to six different populations with different eco-geographic characteristics were sampled and stored in -20 till further use.

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. Morphological characters studied were 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.

DNA extraction and ISSR assay

Fresh leaves were used randomly from one to twelve plants in each of the studied populations. These were dried with silica gel powder. CTAB-activated charcoal protocol was used to extract genomic DNA. The quality of extracted DNA was examined by running on 0.8% agarose gel. For the ISSR analysis, 22 primers from the UBC (University of British Columbia) series were tested for DNA amplification. Ten primers were chosen for ISSR analysis of genetic variability, based on band reproducibility (Table 1). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by the final extension step of 7-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table 1. ISSR primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	17	17	100.00%	0.42	5.66	3.99	4.11
ISSR-2	GGATGGATGGATGGAT	15	12	87.59%	0.35	7.91	4.11	2.13
ISSR-3	GACAGACAGACAGACA	25	25	100.00%	0.24	2.34	4.55	2.55
ISSR-4	AGAGAGAGAGAGAGAGYT	16	16	100.00%	0.21	4.88	3.56	3.22
ISSR-5	ACACACACACACACACC	10	7	77.00%	0.20	5.99	4.99	3.47
Mean		18	16	90.99%	0.29	4.5	4.5	3.5
Total		83	77					

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 CAAT box- derived polymorphism (CBDP) primers

Data analyses

Morphological studies: Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa. For grouping of the plant specimens, The UPGMA (Unweighted paired group using average) ordination methods were used.

ANOVA (Analysis of variance) were performed to show morphological difference among the populations while PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations. PAST version 2.17 (Hammer *et al*. 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses: ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. The discriminatory ability of the used primers was evaluated by means of two parameters, polymorphism information content (PIC) and marker index (MI) to characterize the capacity of each primer to detect polymorphic loci among the genotypes. MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β) (Heikrujam *et al.*, 2015). The number of polymorphic bands (NPB) and the effective multiplex ratio (EMR) were calculated for each primer. Parameters like Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al.*, 2005; Freeland *et al.*, 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, which takes the values of 1-(2x (0.5-p)), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software . Nei's genetic distance among populations was used for Neighbour-Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al*. 2011, Huson and Bryant, 2006). Mantel test checked the correlation between the geographical and genetic distances of the studied populations. These analyses were done by PAST ver. 2.17 software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 were used to show the genetic difference of the populations. Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers the equal amount of gene flow among all populations.

Results and Discussion

Species identification and inter-relationship

Morphometry: ANOVA showed significant differences (P <0.01) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 70% of the total variation. In the first PCA axis with 48% of the total variation, such characters as corolla shape, calyx shape, calyx length, bract length and leaf shape have shown the highest correlation (>0.7), leaf apex, corolla length, leaf length, leaf width were characters influencing PCA axis 2 and 3, respectively. Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters is presented here (Fig. 1). In general, plant samples of each species were grouped together and formed separate groups. This result shows that both quantitative and qualitative morphological characters separated the studied species into distinct groups. In the studied specimens, we did not encounter intermediate forms.

Species identification and genetic diversity: Five ISSR primers were screened to study genetic relationships among *Malva* species; all the primers produced reproducible polymorphic bands in all six *Malva species*. A total of 83 amplified polymorphic bands were generated across 6 *Malva* species. The size of the amplified fragments ranged from 100 to 3000 bp. The highest and lowest number of polymorphic bands was 25 for ISSR-3 and 7 for ISSR-5, on an average of 16 polymorphic bands per primer. The PIC of the 5 ISSR primers ranged from 0.20 (ISSR-5) to 0.42

(ISSR-1) with an average of 0.29 per primer. MI of the primers ranged from 2.13 (ISSR-2) to 4.11 (ISSR-1) with an average of 3.5 per primer. EMR of the ISSR primers ranged from 3.56 (ISSR-4) to 4.99 (ISSR-5) with an average of 4.5 per primer (Table 2). The primers with the high EMR values were considered to be more informative in distinguishing the genotypes.

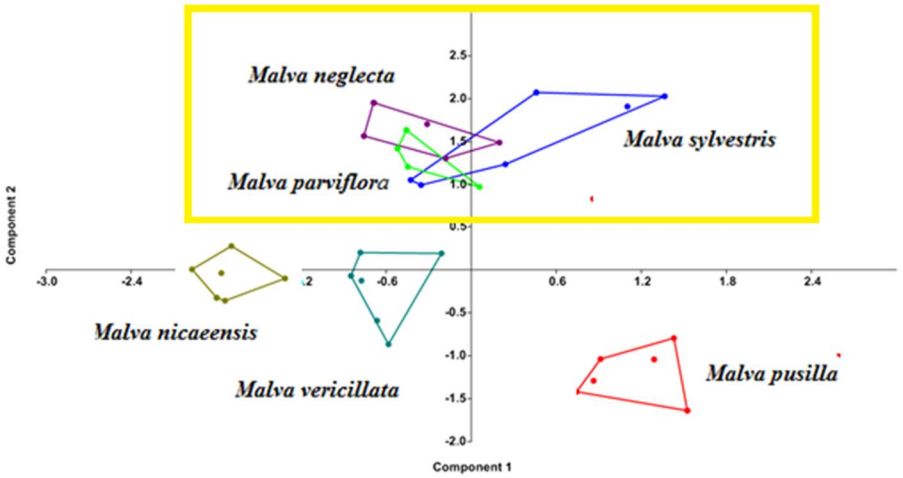

Fig. 1. PCA plots of morphological characters revealing species delimitation in the *Malva* species

Table 2. Genetic diversity parameters in the studied *Malva* species. Abbreviations:

SP	N	Na	Ne	I	He	UHe	%P
Malva neglecta Wallr.	15.000	0.158	1.080	0.304	0.30	0.31	44.50%
Malva parviflora L.	22.000	0.222	1.325	0.231	0.18	0.23	22.1%
Malva pusilla Sm.	13.000	0.167	1.062	0.24	0.224	0.213	39.73%
Malva sylvestris L.	10.000	0.499	1.267	0.12	0.101	0.19	19.46%
Malva vericillata L.	20.000	0.161	1.134	0.372	0.32	0.36	54.15%
Malva nicaeensis All.	10.000	0.345	1.018	0.25	0.20	0.20	49.22%

(N = number of samples, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

The genetic parameters were calculated for all the six *Malva* species amplified with ISSR primers (Table 2). Unbiased expected heterozygosity (*H*) ranged from 0.19 (*Malva sylvestris*) to 0.36 (*Malva vericillata*), with a mean of 0.26. A similar pattern was observed for Shannon's information index (*I)*, with the highest value of 0.37 observed in *Malva vericillata* and the lowest value of 0.12 observed in *Malva sylvestris* with a mean of 0.25. The observed number of alleles (*N*a) ranged from 0.15 in *Malva neglecta* to 0.499 in *Malva sylvestris.* The effective number of alleles (*N*e) ranged from 1.018 (*Malva nicaeensis*) to 1.325 (*Malva parviflora*).

ANOVA test showed significant genetic differences (P = 0.001) among studied species. It revealed that 83% of total variation was among species and 17% was within species. Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.44, P =

0.001) and test values (0.311, P = 0.001). These results revealed a higher distribution of genetic diversity among *Malva* species compared to within species.

Different clustering and ordination methods produced similar results. Here, the UPGMA clustering are presented (Fig. 2). In general, plant samples of each species belonging to a distinct section, were grouped together and formed a separate cluster. This result shows that molecular characters studied can delimit *Malva* species in two major clusters or groups. In the studied specimens we did not encounter intermediate forms. In general, two major clusters were formed in the UPGMA tree (Fig. 2), Populations of *M. neglecta; M. parviflora* and *M. sylvestris* were placed in the first major cluster and were placed with great distance from the other species. The second major cluster included two sub-clusters. Plants of *M. pusilla* comprised the first sub-cluster, while plants of *M. vericillata* and *M. nicaeensis* formed the second sub-cluster.

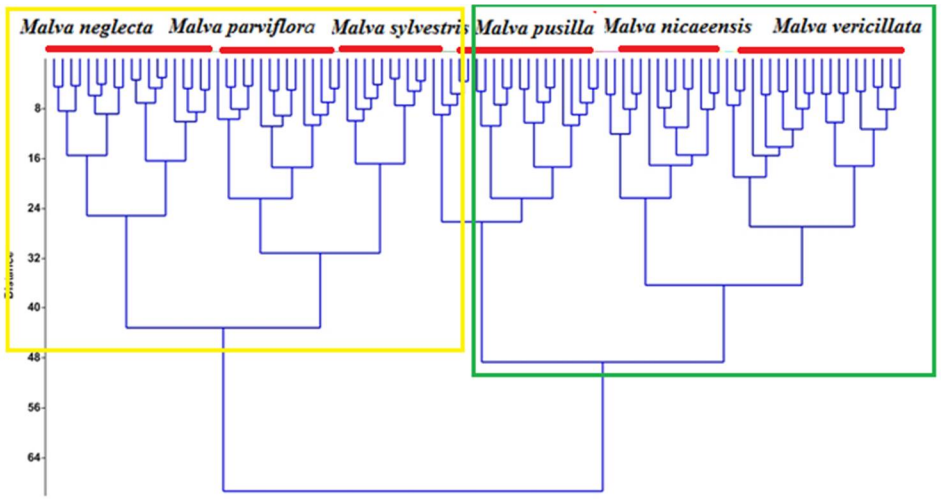

Fig. 2. UPGMA tree of ISSR data revealing species delimitation in the *Malva* species.

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In general, relationships obtained from ISSR data agrees well with species relationship obtained from morphological. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. These results indicate that ISSR molecular markers can be used in *Malva* species taxonomy. The Nm analysis by Popgene software also produced mean Nm= 0.256, which is considered a very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.83, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Malva* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table not included). The results showed that the highest degree of genetic similarity (0.90) occurred between *M. neglecta* and *M. sylvestris.* The lowest degree of genetic similarity occurred between *M. parviflora* and *M. vericillata* (0.73). The low Nm value (0.256) indicates limited gene flow or ancestrally shared alleles between the species studied and indicates high genetic differentiation among and within *Malva* species.

Genetic diversity is an important role in the biology of the long-term evolution of a taxon or a population. The basis of existence, growth, and evolution of taxon. Thus, the study of the genetic diversity of taxon is fundamental to recognize the taxonomy, origin, and evolution of taxon. Moreover, such research will provide a theoretical basis for germplasm resource conservation, development, utilization, and breeding (Bi *et al.*, 2021; Duan *et al*., 2022; Guo *et al.*, 2021; Guo, *et al.*, 2022).

The present research revealed interesting data about genetic variability, genetic stratification and morphological divergence in *Malva* in all parts of Iraq. The degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/cross-breeding brings about higher level of genetic variability in the studied taxon (Li *et al.*, 2021; Sun *et al.*, 2021; Xu *et al*., 2021; Zhang *et al.*, 2022). PIC and MI characteristics of a primer help in determining its effectiveness in genetic diversity analysis. Sivaprakash *et al.* (2004) suggested that the ability of a marker technique to resolve genetic variability may be more directly related to the degree of polymorphism. Generally, a PIC value between zero to 0.25 suggest a very low genetic diversity among genotypes, between 0.25 to 0.50 shows a mid-level of genetic diversity and a value ≥0.50 suggests a high level of genetic diversity (Tams *et al.*, 2005). In this research, the ISSR primers' PIC values ranged from 0.21 to 0.42, with a mean value of 0.29, which indicated a mid-level ability of ISSR primers in determining genetic diversity among the species of *Malva*. All of 5 primer pairs showed a good polymorphism in the taxa of *Malva.* A total 83 alleles were recognized for the studied species. The total number of bands per primer ranged from 7 to 25 polymorphic bands and the mean of the allele number in loci was 16.

In most studies, population size is limited to several vegetative accession (Uotila, 1996). This population could be showed genetic drift, whose effects are observed in the high level of F_{IS} and low level of genetic diversity. The isolation of the population and absence the gene flow led to the fragmentation of the *Malva* population. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.*, 2006). There are two reasons for the positive correlation between genetic diversity and population size (Leimu *et al.*, 2006). The first reason is a positive correlation could imply the presence of an extinction vortex, where the drop-in population size lowers genetic diversity, which leads to inbreeding depression. The second reason is the fact that plant fitness differentiates populations based on variations in habitat quality (Vergeer *et al.*, 2003; Peng *et al*., 2021).

According to Booy *et al.* (2000), low levels of genetic diversity could reduce plant fitness and restrict a population's ability to respond to changing environmental conditions through selection and adaptation. Genetic diversity (17%) was obtained within populations, whereas 83% of genetic variation was obtained between the evaluated populations. One of the key factors determining the distribution of genetic variation is the breeding system in plant species (Duminil, 2007). Couvet (Booy *et al.*, 2000) revealed that one migrant per generation cannot be existed to guarantee the long-term survival of small populations and that the number of migrants is demonstrated through life history characters and population genetics (Vergeer *et al.*, 2003).

There are two hypotheses for the absence of differences between isolated populations. The first hypothesis explained that genetic diversity within and between populations demonstrates gene flow processes, which led to the fragmentation of larger populations (Dostálek *et al.*, 2010). The second hypothesis presented that geographically proximate populations are more efficiently connected through gene flow than populations separated by a greater distance.

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 exploring the genetic relationships and diversity within and

among the population and a 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) have 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) using the AFLP (Amplified fragment length polymorphism) marker explored the phenetic relationships and diversity within and between 13 species under five genera of Malvaceae. The primary objective of the study was to evaluate the taxonomic potential, usefulness and applicability of the AFLP marker system to reconstruct genetic relationships at an 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. 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 clusters representing the 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.

In conclusion, the results of this study showed that to evaluate the genetic diversity of the *Malva* genus, the primers derived from ISSR were more effective than the other molecular markers. Also, *Malva* species were clearly separated from each other in the dendrogram and PCA, indicating the higher efficiency of ISSR technique in *Malva* species identification.

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