J Genet Resour2020;6(2): 157-171 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2020.18739.1190*

Evaluation of Genetic Diversity in Iranian Violet (*Viola* spp) Populations Using Morphological and RAPD Molecular Markers

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ARTICLE INFO

Article history:
Received 09 April 2020
Accepted 14 June 2020
Available online 25 June 2020

Keywords:
DNA extraction
Classification
Cluster analysis
Molecular variance
UPGMA

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p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Recognition of genetic reserves and desirable genes is the basis of breeding programs. So far, in Iran, due to the lack of recognition of genetic resources, a considerable breeding program has not been done on native plants. The study of the genetic diversity of violets as a native plant with ornamental and medicinal uses is the great importance in advancing the breeding goals of this plant. So, in the present study, nine populations of *Viola spp*. from different regions of Iran were used for evaluation of inter and intra-population genetic diversity with RAPD marker, and eight populations of them were used to evaluate morphological, vegetative, and reproductive characteristics. From 11 used primers, 145 bands which showed high resolution and their length was between 250 to 3000 base pairs, were counted and used for RAPD analysis. According to the cluster analysis using the JACCARD similarity coefficient and UPGMA method, significant differences were found among populations. Molecular analysis of variance showed 77 and 23% inter and intra population genetic diversity, respectively. Principal component analysis classified effective characteristics in 6 groups which justified 89.62% of total changes and in the cluster analysis of morphological traits, populations were classified into three groups in distance 10. The results of our molecular and morphological analysis showed considerable diversity among violet populations in Iran, which can be used in future breeding programs.

Please cite this paper as: Abolghasemi S, Naderi R, Fattahi Moghadam MR. 2020. Evaluation of genetic diversity in Iranian Violet (*Viola* spp) populations using morphological and RAPD molecular markers. *J Genet Resour* 6(2): 157-171. doi: 10.22080/jgr.2020.18739.1190

Introduction

Viola genus is one of the largest genera of the Violaceae family comprising 525-600 species which grows in most areas of the world and is divided into 14 sections and many sub-sections (Ballard *et al.*, 1999; Yockteng *et al.*, 2003). Violaceae family is classified based on the following characteristics: lack of stem, short and thick rhizome, rooting stolon, stipule existence, round or sharp or beak-shaped sepal, straight or beak-shaped stigma, seed shape (Mereda *et al.*, 2008). Classification of *Viola* in Europe is done according to capsule morphology (round, without explosion, without tail) which includes nearly 25 native species in temperate regions of Europe and North Africa. Viola section is one of the largest groups of the Violaceae sub-family and is classified into five sub-sections: *Viola*, *Rostratae* ،*Stolonosae* ،*Adnatae*, *Boreali*, *Americana*, *Sororia* which is native to North America (Marcussen, 2006). *Viola* sub-section is classified into two classes: *Viola* class with species of *odorata*, *alba*, and *suavis*, and Eflagellatae class with species of *hirta* and *collina* which have been classified based on stolon existence or absence of stolon (Becker, 1925; Gams, 1926). Around 30 species have been identified in north and northwest of Iran of which 19 species are native to Iran. The most important *Viola* genus medicinal species are *Viola tricolor*, *V. arvensis*, *V. baoshanensis*, and *V. odorata* (Tutin *et al.*, 1964; Mozafarian, 1996; Karimi, 2002). *V. odorata* species which is also

called sweet violet has many applications in the perfume industry and its secondary metabolite have anti-inflammatory, sedative, anti-poisoning, and HIV anti-virus (Drozdova and Bubenchikov, 2004). A combination of karyology, morphology and molecular experiments such as DNA sequencing (Yockteng *et al.*, 2003) and RAPD (Auge *et al.*, 2001), allozyme (Marcussen, 2003) and AFLP markers (Eckestein *et al.*, 2006) has led to clarification of the phylogenetic relationship of intra genus and interspecies of violet.

Violet is an annual or perennial plant with alternate, serrated and congressional leaves with serrated or simple stipule which sometimes grows and gets leaf-shaped, irregular, single, and sometimes self-pollinated flowers, peduncle with leaflets, five uneven petals, lower petals are sometimes larger than others and contain spur, three-chambered ovary containing many ovules and fruit is a capsule (Khatamsaz, 1991). Genetic diversity is highly important in plant breeding and is needed for making any kind of changes in plant genetic structure and to know the level and type of existing diversity in germplasm to be able to use this diversity according to the desired breeding goals (Vojdni, 1993). Different markers are used to evaluate genetic diversity. The importance of morphological markers is that they are cheap and do not need any special molecular and biochemical techniques (Farsi and Zolali, 2003). DNA markers are more powerful than morphological markers in discrimination (Smith and Smith, 1992). The reason is that DNA markers can show the differences among coding rows and adjacent sequences in the genome in addition to differences existing in coding rows. Furthermore, DNA-based markers by developing an unlimited number of markers and removing environmental factors effects could dispel many of the problems related to morphological markers (Shokrpour *et al.*, 2008). RAPD markers which are based on DNA fragments replication by nonspecific primers using polymerase chain reaction have been under special attention in molecular studies especially the evaluation of genetic diversity due to the lack of need to primary information about DNA sequence for designing primers, the possibility of simultaneous evaluation of several places in sample genome, lack of need to the probe, radioactive material, low cost, application and the speed of performance (Williams *et al.*, 1990). To identify the intra and interspecies differences of clone populations of violets native to Germany central forests, six RAPD primers were applied. According to the results of scoring 45 bands, the average ratio of detectable populations and average index of Simpson diversity were 0.93 and 0.99, respectively. In the mentioned study, intrapopulation diversity of violets has been estimated highly which is probably due to the spring cross-pollination of casmogame flowers (Auge *et al.*, 2001). To classify of *Patellares* sub-section which is one of the best parents of violet in the breeding and hybridization programs due to the tolerance to the high temperature, 13 RAPD markers were implicated. OPBO2 primer could classify violet species that corresponded highly to the morphological findings and RAPD analysis was introduced as a useful, quick, and simple method (Oh *et al.*, 1998). To determine the interspecies differences of *Viola suavis*, AFLP molecular marker was used and classification analyses showed that intra spices white flower violets were classified into two groups which indicate the far distance of these populations geographical origin (Mereda *et al.*, 2008). Culley *et al.* (2007) used the ISSR marker to estimate the effect of activity and geographical division effects on the diversity of Ohio city violets. The results showed that the population diversity has been kept at a high level (80.7%), geographical distance is significantly correlated with genetic distance, and inter and intra-population diversity was estimated at 69.1% and 22%, respectively. The populations were classified in separate clusters and showed a high distance towards other regions. Genetic diversity and classification studies on violet using isozyme markers have been performed by different researchers (Marcussen and Nordal, 1997; Marcussen, 2003; Marcussen and Borgen 2011). In a research study performed on the diversity of *V. odorata* collected from South Europe, North Africa, and West Asia, 28 isozyme markers were applied. Clustering of populations showed that Scandinavia and England populations had high homogeneity with the west European population which displays the same origin of these populations (Marcussen, 2006). To classify and

discriminate different populations, samples were collected from Iran, northwest Africa, East Europe, Turkey, and Azerbaijan, and morphological and isozyme markers were used. Populations were clustered in three groups and the population collected from the Golestan province of Iran was clustered in *Viola alba subsp. sintenisii* (Mereda *et al.*, 2011). The majority of studies on violet have been on the essence and medicinal characteristics. Collecting genetic materials and studying their diversity, resolution, and comparison can efficiently help the violet breeding. So, in the present study, the morphological and genetic diversity of Iranian native populations of violet were studied using morphological and RAPD markers, respectively.

Materials and Methods

In the spring of 2012, nine natural habitats of wild violet in Mazandaran, Tehran, Alborz, Kermanshah, and Hamedan provinces were identified and the collected samples were transferred to the greenhouse (Table 1).

Table 1. The climate of different wild violet sampling regions in Iran (According to the information of Iran meteorology calendar of 2008).

Population No.	Sampling region	Yearly relative humidity (%)	MT of the coldest month of the year (ºC)	MT of the warmest month of the year (ºC)	Mean annual rainfall (mm)	Altitude above sea level (m)
1	Ramsar-Mazandaran	80	3.9	26.6	1257.6	-20.0
2	Sisangan-Mazandaran	83	7.9	25.2	1281	-20.0
3	Khalkhal-Ardabil	62	-9.3	20.1	246.0	1796
4	Varian-Alborz	47	-5.7	23	168.0	1312.5
5	Lavasanat-Tehran	46	-2.5	27.2	410.5	2000
6	Chalous-Mazandaran	82	1.7	21.1	1257.6	73
7	Kheiroud-Mazandaran	83	7.9	25.2	1281	-20.0
8	Nahavand-Hamedan	44	-6.1	39.2	263.0	1680.9
9	Kermanshah	39	-2.9	29.0	305.1	1318.6

MT: Mean temperature

Evaluation of morphological features

Thirty-seven characteristics (19 vegetative, 13 reproductives, and five relative characteristics) were measured on 80 plants collected from 8 populations (Table 2, Fig. 1). Characteristics of stipule, lamina, and petiole were measured on three mature and developed leaves in each plant, and characteristics of the flower stem, calyx, and corolla were measured on two large flowers.

DNA extraction

For extraction of DNA from the violet plant, young leaves were collected, wrapped in aluminum foils, and placed on ice to transfer to the lab. The DNA was then extracted following the method of Doyle *et al.* (1990) with one modification including the addition of 3% CTAB to the extraction buffer. After the extraction process, DNA existence, concentration, and quality were assayed in samples. Three methods for determining the quality and quantity of samples DNA were applied. In the present study, two techniques of agarose gel electrophoresis and NanoDrop (Thermo- Nanodrop 1000) were used for evaluating the quality and quantity of DNA.

Polymerase chain reaction (PCR) conditions

Each reaction mix with the final volume of 15 µl included 2.5 µl template DNA (10 ng/ µl), 1.5 µl of random primer of RAPD with the concentration of 10 ng/µl, 4 µl sterile distilled water, 7.5 µl Polymerase Master Mix Red – Ampliqon Tag DNA kit. For DNA polymorphism evaluation among studied populations, 40 number of 10 nucleotide RAPD primers purchased were used for primary screening. Finally, 11 primers showed high polymorphism that was selected for the molecular experiment of RAPD (Table 3).

Table 2. Characteristics studied in violet populations.

Characters	Character explanation
Stolons	
StAL	The maximum length of aboveground stolon (cm)
StP	Violet pigmentation of stolons 0 absent; 1 present
Laminas and petioles	
LL	Lamina length (cm)
LW	Lamina width (cm)
LL1	Lamina length from the base to maximum width(cm)
LSL	Lamina sinus depth (cm)
LSW	Lamina sinus width (cm)
LCN	Number of crenulae along both lamina margins (lamina dentations)
LAA	Lamina apex angle (degree)
LSA	Lamina sinus angle (degree)
AE	Stationary angle (degree)
Laminas and petioles	
SL	Stipule length (mm)
SW	Stipule width (mm)
SFN	Number of fimbriae (= glandular fimbriae, non-glandular fimbriae, and sessile glandule) along both stipule margins
SFL	Maximum fimbriae length on stipule (mm)
SGN	Number of glandular fimbriae along both stipule margins
SYGN	Yellow or yellowish-brown glandular fimbriae on stipule (including yellow or yellowish-brown sessile glandule) 0 absent; 1 present
SBGN	Blackish glandular fimbriae on stipule (including blackish sessile glandule) 0 absent; 1 present
LL/LW	Lamina length/lamina width
LW/LSW	Lamina width/lamina sinus width
LL1/LL	Lamina length from the base to maximum width/lamina length
LSL/LL	Lamina sinus depth/lamina length
Peduncles	
PL	Peduncle length (cm)
PL1	PL1 Peduncle length below bracteoles (cm)
PL1/PL	Peduncle length below bracteoles/peduncle length
Calyx (sepals)	
KAL	Anterior sepal length (mm)
KAW	Anterior sepal width (mm)
Corolla (petals)	
CPL	Posterior petal length (mm)
CPW	Posterior petal width (mm)
CPL1	Posterior petal length from the base to the first maximum width
CLL	Lateral petal length (mm)
CLW	Lateral petal width (mm)
CAL	Anterior petal length (including spur) (length of flower) (mm)
CSL	Spur length (mm)
CSP	Spur colour: 0 white; 1 pale blue or (bluish-)violet; 2 deep violet
CPSP	Pigmentation of the corolla in contrast to pigmentation of spur: 0 spurs paler than corolla; 1 spur the same color as the corolla; 2 spurs darker than corolla
CPL1/CPL	Posterior petal length from the base to the first maximum width/posterior petal length

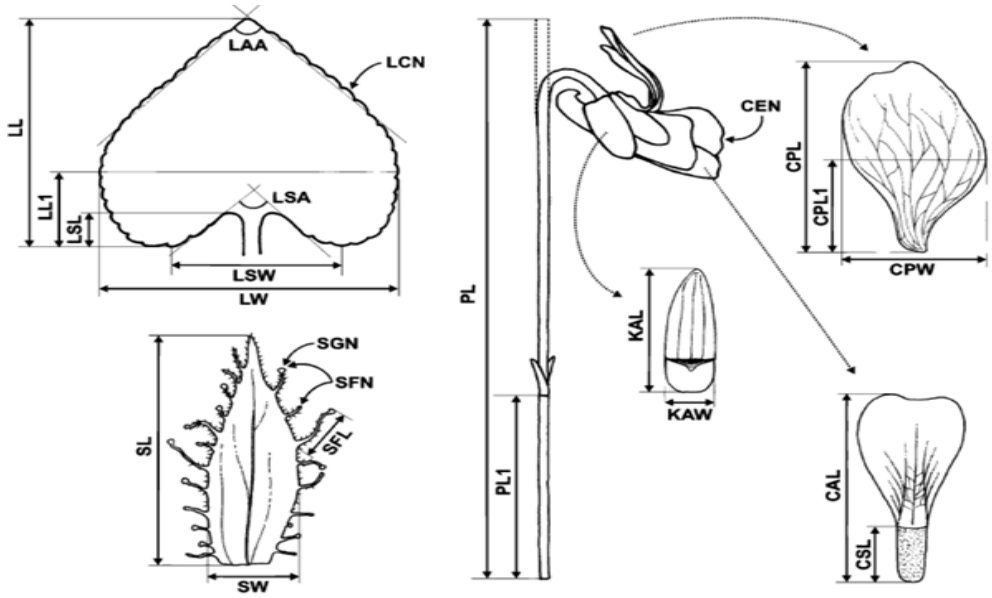

Fig. 1. The measured morphological features were taken from Hodalova *et al.* (2008).

Table 3. Information on used random primers and their obtained bands.

Code	Primer	Primer sequence (5'→3')	Total bands number	Polymorph bands number	Polymorphism percent (P%)	The resolution power of primers (Rp)
1	BD-06	AAGCTGGCGT	16	4	25	2.263
2	BD-08	CATACGGGCT	8	5	62.5	1.947
3	BD-09	CCACGGTCAG	23	20	86.95	7.526
4	BD-13	CCTGGAACGG	11	7	63.63	5
5	BD-15	TGTCGTGGTC	11	7	63.63	1.894
6	BD-19	GGTTCCTCTC	14	4	28.57	3.157
7	BE-05	GGAACGCTAC	10	5	50	1.947
8	BE-06	CAGCGGGTCA	14	10	71.42	5.473
9	BE-14	CTTTGCGCAC	11	6	54.54	2.947
10	BE-18	CCAAGCCGTC	18	14	77.77	6.947
11	BE-20	CAAAGGCGTG	9	6	66.66	4.368
Sum	-	-	145	84	-	43.469
Mean	-	-	13.18	7.63	59.15	3.95

PCR was performed using a thermocycler (Bio Rad-C1000 Touch) and cycles described in Table 4. After doing PCR, the product was loaded in 1.2% agarose gel wells prepared using Tris-Boric acid-EDTA (TBE) buffer, and gel electrophoresis was run at a voltage of 80V for 120 min. To calculate the size of the obtained fragments, 1 Kb marker (Fermentas) was added to the first and last well in each gel. After electrophoresis, the gel was soaked in 0.5 microgram/mL ethidium bromide for 20 min, washed with distilled water for 15 min and observed and photographed replicated DNA strips with Gel Doc (UVP-Bio Doc-It^{Tm} System) under UV.

Table 4. Time and temperature needed for three steps (denaturation, annealing, and extension) in each of the PCR Thermocycling.

Cycle	Repeat no.	Stage	Temperature (℃)	Time (min)
1	1	Denaturation	95	4
2	5	Denaturation	94	30
		Annealing	37-40.5	45
		Extension	72	2
3	30	Denaturation	94	30
		Annealing	37	45
		Extension	72	2
4	1	Final extension	72	10

Statistical analyses

Morphological characteristics were analyzed using SAS version 9.3. Correlation and principal component analysis (PCA) was performed by using SPSS version 17.

For analyzing data according to the existence (1) and absence (0) of PCR products, data were ranked and similarity coefficients are calculated according to the simple matching coefficients. Then these coefficients were used for developing dendrogram based on UPGM with NTSYS pc 2.02e. The population distance matrix was calculated using Nie (1973) method. In this evaluation, observed and effective alleles number (Kimura and Crow, 1963) and Shannon Information Index (Lewontin, 1972) were calculated for each population. The genetic diversity index of Nie (h), Shannon Information Index (I), ne and na molecular indices, polymorphism percent (P%), and resolution power (Rp) was calculated.

Results and Discussion

Morphological marker analysis

On the ground, stolon length is of characteristics that are considered in the traditional classification of violet so that *V. odorata, V. suavis,* and *V. alba* were classified as species with stolon and *V. collina, V. hirta, V. ambigua* were classified as without stolon (Gams, 1926; Marcussen, 2003). In the present study, all collected populations had on ground stolon except some plants of the Varian population while the longest stolon was related to Ramsar and Sisangan populations. One of the important characteristics which make the differentiation among *V. alba* is the violet pigment of stolon, leaf, capsule (Hodalova *et al.*, 2008).

In the present study, Ramsar, Sisangan, Kheiroudkenar, Nahavand, and Kermanshah populations included violet pigment in their organs while Khalkhal and Varian populations lack violet pigment. Flower length (the length of the front petal with spur) showed the highest and lowest amounts in Kermanshah (28.75) and Khalkhal (8.14) populations, respectively. Peduncle length up to the leaflet is an important characteristic of making differentiation among populations that the highest (0.739 mm) and lowest (0.258 mm) amounts were related to Ramsar and Sisangan populations, respectively.

Principal component analysis (PCA)

The results of the PCA are shown in Table 5. The relative variance of each component displays the importance of that component in the variance of all studied features and is described as the percent. Here, six main and independent components with special amounts of more than 0.6 could generally explain 89.62% of the total variance. Populations were classified in the first component considering the characteristics of color, spur, corolla color compared to spur color, length to width ratio of leaf lamina, leaf sinus depth to leaf length ratio, peduncle length up to leaflet to peduncle length ratio, and leaf sinus angle which included 32.03% of the variance. The characteristic of leaf fluff length was mentioned as the differentiation factor of *V. alba* three subspecies by Marcussen (2003).

Populations considering the characteristics of flower length, axillary and dorsal petal length, axillary and dorsal petal width, and the number of stipule fimbria were placed in the second component which could justify 26.429% of the variance. According to the PCA results (Table 5), some characteristics related to flower, leaf, and stipule which were placed in the first and second components, played the most important role in the discrimination of populations that in sum devoted almost 58.459% of variance to themselves. Features like leaf lamina width and length, stipule width and length, and leaf sinus depth were placed in the third component which could justify 16.484% of the total variance. The fourth component comprised characteristics like leaf angle with a stem which included 6.447% of the variance. The fifth and sixth component groups justified 4.126 and 4.111% of the total variance, respectively. The goal of this procedure is justifying current variances in some of the primary variables using a lower number of variables (indices) (Guetrin and Bailey, 1970).

Correlation coefficients

When measuring a feature is complicated, expensive, and time-consuming, other features with a highly significant correlation can be used for indirect measurement of it.

Table 5. The results of principal component analysis.

Coefficient Factors	1	2	3	4	5	6
Cumulative variance percentage	32/03	58.45	79.94	81.39	85.51	89.62
Characters	1	2	3	4	5	6
StAl	-0.408	-0.023	-0.103	0.033	-0.288	0.803
StP	0.427	0.320	-0.051	0.064	-0.662	0.217
LHL	0.848	0.063	-0.045	-0.140	-0.315	0.015
LL	-0.531	-0.056	0.756	-0.034	-0.153	0.037
LW	-0.510	-0.119	0.709	-0.294	-0.018	-0.003
LL1	-0.301	0.147	0.893	-0.024	0.038	0.156
LSL	-0.162	0.216	0.931	0.028	-0.094	0.037
LSW	-0.092	0.488	0.789	-0.062	0.062	-0.026
LCN	-0.227	-0.722	0.059	-0.535	0.189	0.127
LAA	-0.647	-0.354	0.087	-0.390	0.385	-0.102
LSA	-0.877	-0.207	0.056	-0.040	-0.106	0.011
AE	-0.371	-0.146	-0.127	-0.775	0.092	0.053
SL	-0.157	0.296	0.853	0.066	0.073	-0.073
SW	0.117	0.196	0.785	0.452	0.046	-0.107
SFN	0.041	-0.840	0.268	-0.134	0.212	0.0353
SFL	0.247	0.407	0.615	0.448	-0.001	-0.175
SGN	0.166	-0.722	0.166	-0.082	0.223	0.545
SYGN	0.397	-0.626	0.211	-0.408	0.282	0.238
SYBN	0.560	-0.421	0.032	0.365	0.053	0.405
PL	-0.619	0.145	0.066	0.509	0.197	0.126
PL1	-0.343	0.476	-0.348	-0.098	0.569	-0.003
KAL	0.117	0.757	0.460	-0.004	-0.089	0.247
KAW	0.322	0.709	0.328	0.377	-0.283	-0.067
CPL	-0.005	0.926	0.179	0.146	0.005	-0.051
CPW	0.223	0.784	0.290	0.050	-0.075	0.048
CPL1	0.262	0.893	0.210	0.016	-0.027	0.066
CLL	0.145	0.923	0.195	-0.023	0.066	0.002
CLW	0.326	0.821	0.343	0.059	0.039	0.050
CAL	0.056	0.912	0.224	0.130	0.171	-0.073
CSL	0.463	0.733	0.214	-0.171	0.201	0.088
CSP	0.969	0.093	-0.031	0.128	-0.078	-0.031
CPSP	0.962	0.151	-0.085	0.109	-0.053	-0.077
LL/LW	0.968	0.148	-0.097	0.108	-0.052	-0.045
LW/LSW	0.908	-0.153	-0.280	0.023	-0.042	0.036
LL1/LL	0.970	0.151	-0.095	0.093	-0.039	-0.042
LSL/LL	0.969	0.152	-0.092	0.095	-0.042	-0.045
PL1/PL	0.968	0.157	-0.113	0.083	-0.033	-0.046
CPL1/CPL	0.967	0.165	-0.094	0.091	-0.045	-0.043

Simple correlation coefficient of features showed that leaf lamina length and lamina sinus depth (r = 0.911), number of stipule fimbria and fimbria stipule containing gland (r = 0.914), leaf tip angle and spur color (r = 0.746), lateral and posterior petal length (r = 0.917), anterior sepal length and anterior petal width (r = 0.846), leaf lamina sinus angle and the ratio of length to leaf lamina width (r = 0.854), stipule length and leaf lamina width (r = 0.615), flower length (frontal petal length along with spur) and number of leaf margin cuts (r = 0.686), leaf sinus width and frontal sepal length (r = 0.726), number of leaf margin cuts and number of stipule fimbria (r = 0.751) were significantly ($p < 0.01$) correlated (Table 6). An increasing number of stipule fimbria is accompanied by increasing the number of fimbria with glands while changing spur color from violet to white, the leaf tip angle gets tighter. Violets with deeper leaf sinus had a longer stipule toward violets with lower sinus depth. Violets with more leaf lamina margin cuts had stipules having many fimbriae and smaller flowers. Dark violet flowers had longer and broader leaves. Violet flowers collected from Ramsar and Chalous road had small and thin leaves and white flowers. Longer stipules were related to violets with longer leaves.

Table 6. The correlation coefficient among some studied features.

Characters	StN	LL	LW	LL1	LSL	LSW	LCN	LSA	SL	SFN	SGN	KAL	CAL	CLW
StN	1													
LL	0.158	1												
LW	0.097	0.930**	1											
LL1	0.174	0.844**	0.790**	1										
LSL	0.027	0.786**	0.718**	0.911*	1									
LSW	-0.91	0.588*	0.573**	0.807**	0.841**	1								
LCN	0.127	0.243	0.429**	0.059	-0.111	-0.255	1							
LSA	0.382*	0.561**	0.547**	0.267	0.197	0.016	0.326	1						
SL	0.076	0.615**	0.551**	0.826**	0.847**	0.798**	-0.15	0.047	1					
SFN	0.185	0.219	0.302	0.156	0.05	-0.212	0.751**	0.165	-0.048	1				
SGN	0.313	0.002	0.067	0.055	-0.026	-0.231	0.62**	-0.002	-0.09	0.914**	1			
KAL	-0.096	0.27	0.177	0.541**	0.588**	0.736**	-0.542**	-0.211	0.559**	0.448*	-0.357	1		
CLW	-0.225	0.038	-0.033	0.29	0.438*	0.662**	-0.657**	0.451*	0.489**	-0.561**	-0.426**	0.846**	1	
CAL	-0.19	0.068	0.001	0.294	0.407*	0.597**	-0.686**	-0.238	0.474*	-0.703**	-0.629**	0.752**	0.863**	1

Wider leaf sinus angle accompanies with lower leaf length to width ratio and as a result, leaves with wider sinus angle have broader leaves and wider leaf sinus accompanies with longer frontal sepal length. Most of the characteristics related to reproductive organs such as frontal and dorsal petal length and sepal length had a high positive correlation. In the separation of Balkan Island countries native violet populations, Mereda *et al.* showed the highest correlation in characteristics of flower corolla color and corolla color in comparison with spur color (Mereda *et al.*, 2011).

Cluster analysis

In the present study, cluster analysis was carried out according to six main factors that showed the highest variance (89.62%). According to Fig. 2, the distance of 10 populations was divided into 3 groups.

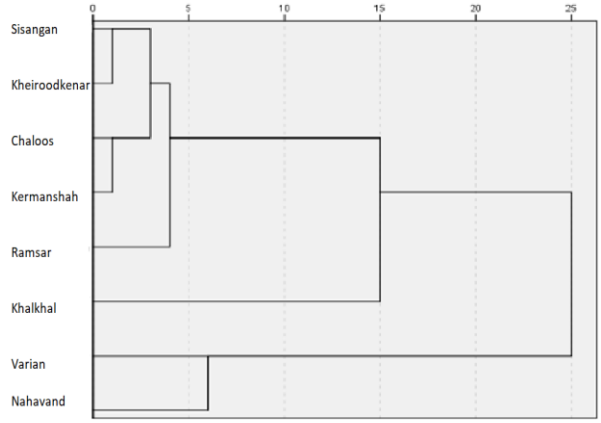

Fig. 2. Clustering of violet populations based on morphological characteristics.

Ramsar, Sisangan, Chalous road, Kheiroudkenar, and Kermanshah were classified in the first group which has relatively large flowers with white color and violet to pale violet spots at the end of petal, long on ground stolon, sharp leaf angle with stem, low number of leaf indentation and low number of fimbria on both sides of stipule. Ramsar with the longest on ground stolon and the smallest stipule and Sisangan with the longest peduncle and Kermanshah with the longest stipule were classified in this group. The reason for classifying Sisangan and Kheiroudkenar populations next to each other in a cluster may be due to collecting both populations from Mazandaran province and district of Noshahr city that their leaf and flower characteristics are so similar.

The second group comprised the Khalkhal population which showed characteristics like short stolon, vertical angle of leaf tip, and short stipule as well as the shortest and highest number of the fimbria and small dark violet flowers. This population includes the shortest peduncle and the lowest leaf sinus wide and smallest flower. Khalkhal population differs from other regions considering geographical location and climate. It has the highest altitude, very cold winter and cool summer that influence on wild plants characteristics of this region.

The third group included Varian and Nahavand populations with relatively large and broad leaves and a high number of margin cuts, the sharpest leaf tip angle, small and violet flowers, and relatively thin stipule with a high number of fimbria. Nahavand population with the sharpest leaf tip angle and highest number of fimbria with the yellow color gland of stipule and the Varian population with the sharpest leaf static angle and lowest peduncle length up to leaflet to peduncle length are in this group. Mereda and Hodalova evaluated 50 quantitative and qualitative characteristics of 58 populations collected from Slovakia. According to their results, populations were clustered in 6 clusters which were used to classify *Viola* sub-sections (Mereda *et al.*, 2008). In a study on the morphological and molecular characteristics of Iran, Azerbaijan, Europe East, and Turkey violet, populations were clustered into four clusters so that collected samples from Bandargaz, Gorgan province were classified in *Viola alba spp. Sintenisii* species (Marcussen *et al.*, 2005).

Molecular marker analyses

In the present study, RAPD molecular markers were used for evaluation of the intra and inter-population of Iran native violet. From 11 used primers, 145 bands which showed high resolution and their length was between 250 to 3000 base pairs, were counted and used for RAPD analysis. From the total counted bounds for different populations, 61 uniform and 84 (59.15%) bands were also polymorph. Averagely, 13.18 bands obtained per all primers that 7.63 of them showed polymorphism. The lowest (13.8) and highest (23) number of

amplified bands obtained with the primers of BD-08 and BD-09, respectively (Table 3). The highest polymorphism percent was observed using BD-09 and BE-18 primers (89.95% and 77.77%, respectively), while BD-06 showed the lowest (25%) polymorphism percent.

Distance matrix

The similarity or differences among populations were determined by distance matrix and the obtained dendrogram as well (Table 7). In the evaluation of the distance matrix, Nahavand and Kermanshah showed the highest (0.047) similarity among studied populations; the possible reason for this similarity can be the similarity of these two populations harvest regions. The results of the distance matrix table show that Varian and Ramsar populations showed the highest difference (0.893) among studied populations.

Table 7. The distance among 9 studied populations of wild violet.

Populations	Ramsar	Sisangan	Khalkhal	Varian	Lavasanat	Chalous	Kheiroudkenar	Nahavand	Kermanshah
Ramsar	0								
Sisangan	0.711	0							
Khalkhal	0.858	0.526	0						
Varian	0.893	0.316	0.457	0					
Lavasanat	0.784	0.319	0.551	0.124	0				
Chalous	0.456	0.495	0.613	0..416	0.340	0			
kheiroudkenar	0.601	0.206	0.372	0.323	0.246	0.347	0		
Nahavand	0.695	0.378	0.533	0.231	0.231	0.330	0.272	0	
Kermanshah	0.747	0.393	0.552	0.240	0.276	0.351	0.309	0.047	0

The calculated distance among populations based on polymorph bands was in the range of 0.047 to 0.893. In the obtained dendrogram from the population's distance amounts (Fig. 3), the Ramsar population showed the highest difference with other populations so that it was classified in a completely distinct group toward the other populations. Khalkhal population was classified in a distinct group, as well. Nahavand and Kermanshah populations were classified in a group together and then Sisangan and Kheiroudkenar populations that were collected from the same region were classified in a single group. Varian and Lavasanat populations were also classified into distinct groups together. Although the populations of Chalus, Ramsar, and Sisangan are geographically close, they are not in a group. Genetic distances between populations do not correspond to the geographical distance between their natural habitats, so that study conducted in the sunflower showed similar results (Jannatdoust *et al*., 2014). Nahavand and Kermanshah have climatic similarities as they have the same mean temperature of the warmest month of the year, high annual precipitation, and under zero temperature of the cold months of the year.

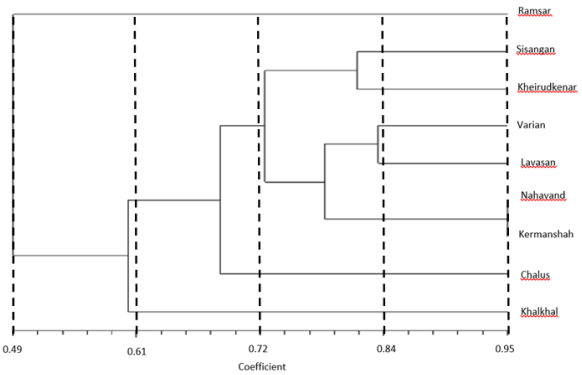

Fig. 3. Classification of 9 studied populations of native violet according to distance matrix obtained from RAPD data.

They are also considered of the country highlands which can justify the little distance between these two regions in the distance matrix, but another reason for this little distance should be searched in the gene content of these two populations. Due to the random performance of the RAPD marker, primers connection to the whole plant genome is completely randomized and the probability of genome exact reading by this marker is not possible.

Collected samples from Sisangan and Kheiroudkenar, close regions in Mazandaran

province, due to the same latitude and longitude, precipitation, temperature, and other climatic characteristics were placed with the little distance in the similarity matrix. Another possibility is that the genetic content of these two populations is similar, so that they may have originated from even one species but show different morphological traits, as shown in a study in Azerbaijan, two violets of the genus *Viola alba* that live next to each other, they have different morphological features (Marcussen, 2003).

A species of violet grows in Khalkhal, one of the coldest regions of the country which has temperate and cold summers and high annual precipitation. It is compatible with the mentioned conditions and can tolerate the low temperature of this region. This population has been also separated into morphological characteristics.

Cluster analysis

According to the results of the cluster analysis, in the distance of 37, populations were classified into four groups (Fig. 4).

The first group includes the Khalkhal population. The second cluster includes Chalous road, Kermanshah, Nahavand, Lavasan, and Varian populations. Populations of Sisangan and Kheiroudkenar are in the third cluster and Ramsar is the fourth cluster. In the distance of 58, populations are clustered in 9 groups. So that in the first cluster, the Khalkhal population, in the second cluster, Chalous road population, in the third cluster, Nahavand and Kermanshah populations, and in the fourth cluster, Varian and Lavasan populations are classified. In the fifth, sixth, and seventh clusters, the Kheiroud population was classified which shows the intra-population diversity of this population. In the eighth cluster, the population of Sisangan and in the ninth cluster, the population of Ramsar was classified. In the distance 100, the individuals of each population were separated from each other except the Ramsar population in which the individuals did not show differences in RAPD analysis due to probably its exclusive vegetative propagation by stolon.

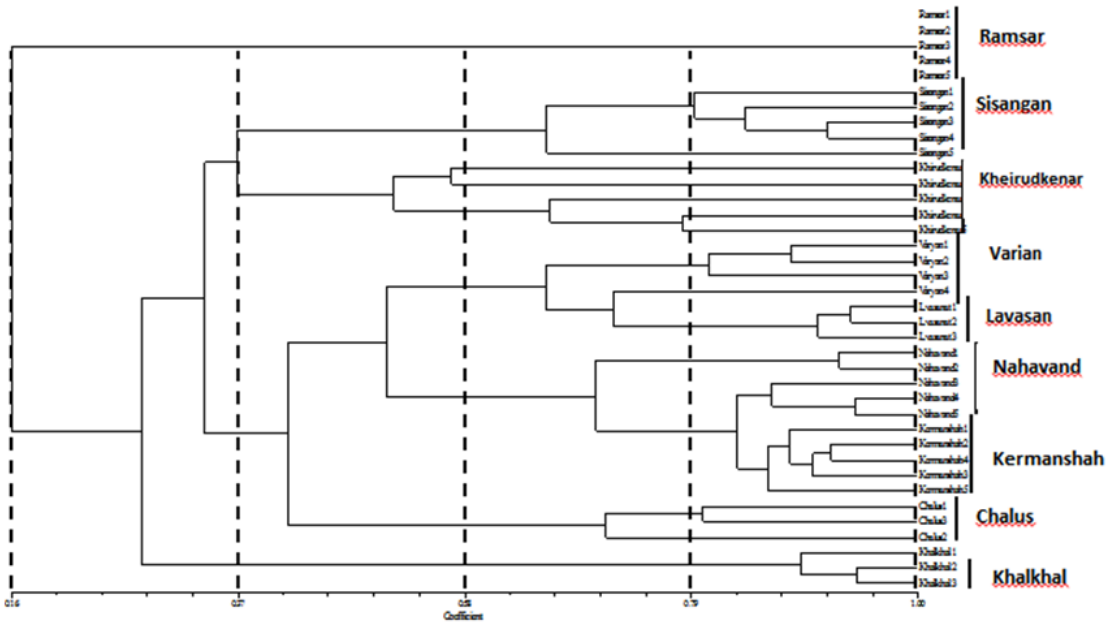

Fig. 4. The results of the cluster analysis of studied violet populations in the RAPD experiment by using UPGMA and JACCARD similarity.

Polymorphism percent

Intra population polymorphism percent showed that there is little difference among intra-population plants of collected violets (Table 8). The highest and lowest individual difference was observed in populations of Kheiroudkenar (30.95%) and Ramsar (0.0%), respectively. The polymorphism percent of zero in the Ramsar population is probably due to the exclusive

vegetative propagation of it by stolon in the region of sampling and/or the small size of this population in Ramsar, self-pollination and vegetative propagation or could be because of limitation of the RAPD technique or wrong sampling in this region, have resulted in the homogeneity of the population with low differentiation.

Table 8. Intra-population polymorphism percent of wild violet.

Violet collected populations	Polymorphism percent
Ramsar	0.0
Sisangan	14.29
Khalkhal	4.76
Varian	15.48
Lavasanat	4.76
Chalous road	21.43
Kheiroudkenar	30.95
Nahavand	19.05
Kermanshah	9.52

Wild violet populations' diversity was evaluated using the genetic diversity index of Nie (h), Shannon Information Index (I), (Table 9). Intra population genetic diversity of Kheiroudkenar (h = 0.112 and I = 0.167) was the highest toward other populations while it was the lowest toward the Lavasan population (h = 0.017 and I= 0.025).

RAPD molecular data analysis of variance

Obtained results from molecular data analysis of variance (AMOVA) show that intra populations variance differences were not significant at 1 and 5% levels and more than 75% of related changes were related to inter populations (Table 10). The results of the distance matrix show that there are high inter-population differences so that the difference between the two populations of Varian and Ramsar was 89%. Geographical distance and gene flow among populations is determining the genetic distance in wild populations.

Table 9. Genetic diversity parameters in studied violet populations.

Population	Number of individuals	na[a]	ne[b]	h[c]	I[d]
Ramsar	5	0.357	1.000	0.000	0
Sisangan	5	0.524	1.076	0.046	0.070
Khalkhal	3	0.488	1.039		
Varian	4	0.619	1.107	0.061	0.090
Lavasan	3	0.440	1.027	0.017	0.025
Chalous	3	0.798	1.148	0.084	0.124
Kheiroudkenar	5	0.810	1.193	0.112	0.167
Nahavand	5	0.714	1.131	0.075	0.110
Kermanshah	5	0.560	1.060	0.035	0.052

[a] na = number of observed allele, [b] ne = number of effective allele, [c] h = Nie genetic diversity index, [d] I = Shannon information index

Table 10. The results of molecular data analysis of variance (AMOVA) of studied violet populations.

Source	Degree of freedom	Sum of squares	Mean of squares	Estimated variance	Variance percent
Inter population	8	461.871	57.734	12.830	77%
Intra population	29	112.550	3.881	3.881	23%
Total	37	574.421		16.711	100%

Due to the low gene flow in self-pollinating species and with some vegetative propagation, the intra-population genetic distance is low while genetic diversity among populations is dispersed, so that in the present research, the intra-population variance is just 23% (Fig. 5). Our results are consistent with the results of research conducted in Ohio (Culley *et al.*, 2007) on wild violets dispersed in the urban environment so that the molecular variance results of that study showed the inter-population distance of 69.1% and intra-population variance of 22%. While gene flow among habitats is cut under the effects of unnatural factors such as habitats destruction due to the excessive harvest, too much grazing, and other factors, the genetic distance among cut

populations increases and genetic erosion will be initiated as a result of homogeneity and population differentiation (Hameric and Godt, 1990).

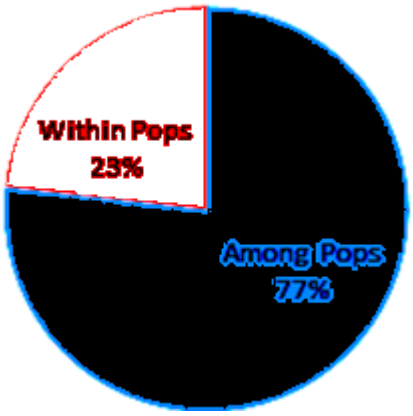

Fig. 5. Molecular variance percent of collected wild violet inter and intra-population.

Evaluation of genetic diversity in populations is complicated due to the interference of several factors such as linkage, crossing, relationship, immigration, and differences among populations individuals (Mohammadi and Perasana, 2003). Generally, these evaluations depend on the number of studied individuals in each population, the number of studied allelic loci, population allelic and genotypic locations, crossing type, and the size of the population (Weir, 1990). In plants which are usually propagated by vegetative procedures, like violet that is typically propagated by using on-ground stolon and rhizomes, very little individual and intra-population differences are observed and the little-observed differences are due to the sexual propagation of cosmogenous flowers in early spring and dispersion of seeds obtained from cross-pollination (Becker, 1910). This also happens in plants like fritillaries which are scarcely self-pollinated but are propagated commercially using vegetative organs (tuber), so that high genetic diversity is observed among populations with far habitat distance while there is little diversity among each population individuals (Koohgard *et al.*, 2011). In cross-pollinating heterozygous plants like thymes, high intra-population diversity is observed that is an opportunity for evolution and compatibility in these populations (Yavari *et al.*, 2012). Genetic variability and differentiation of six populations of *Tilia rubra* from Hyrcanian forests of the north of Iran were analyzed using random amplified polymorphic DNA (RAPD) markers, the results indicate that the genetic differentiation among different *T. rubra* populations is low and the majority of genetic diversity is located within populations (Colagar *et al*., 2013).

Evaluation of plant genetic resources diversity (germplasm) which includes a high chance for useful gene discovery, is of essential prerequisites for programming toward protection, domestication, and sustainable exploitation of them. Despite troubles that have been reported on RAPD molecular markers, they are simple and efficient tools for evaluation and study of plant recourses genetic diversity for which there is no preliminary information on their genomic DNA and the diversity among them.

In the present study, RAPD molecular markers were used for the evaluation of the germplasm genetic diversity of some wild violet populations in Iran. This molecular system revealed the genetic diversity of existing inter and intra-population as well as differentiating populations. Knowing the genetic relationship of wild species is essential for successful and sustainable exploitation of their genetic diversity. Morphologic data could properly classify Iran native violets and show the population's differences. Violets with larger flowers such as Kermanshah and Chalous population and violets with larger leaves such as Kermanshah and Varian populations could be recommended for medicinal and ornamental uses due to the large size of their flower and leaves, while violets with long stolon containing reproductive buds like Sisangan could be recommended for breeding applications due to easy and fast propagation.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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