Morphological And Molecular Identification of Double Flowered Stock (*Matthiola Incana* R. Br) Cultivars With High Fertility

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Research Article

Keywords: Matthiola incana, RAPD, ISSR, cultivar, identification, fertility

Posted Date: June 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-578473/v1

Abstract

Garden stock (*Matthiola incana* R. Br.) is a commercially important horticultural crop owing to its ornamental effect. There are different stock cultivars varied in color and shape, especially flowered phenotype is an essential index evaluating its commercial value, because double flowered cultivars have more brilliant flowers compared to single flowered one. The present work aimed: (1) to make superior cultivars with different colors, high fertility, being capable of early selecting only double flowered seedlings by leaf color and to investigate morphological characteristics and (2) to select RAPD and ISSR primers for the cultivar certification and identification to culture and produce good commercial stock cultivars. Here we obtained new double flowered stock cultivars with different colors including pink, pale pink and white, through outcrossing between “white” cultivar (high fertile but unable to select double flower phenotype) and “pink” cultivar (vice. versa). Among newly obtained stock cultivars, single and double flower seedlings are distinguishable from each other by leaf color, having about 70% of fertility. Moreover RAPD and ISSR markers selected in this study can be applied to identify different stock cultivars in seed production, culture and to establish cultivar certification system.

Introduction

Garden stock (*Matthiola incana* R. Br.) is a commercially important horticultural crop having different types of flowers varied in color and flowered phenotype, belonging to the family *Brassicaceae* (Tatsuzawa et al. 2012).

Due to high ornamental value, many studies associated in culture and breeding of the garden stock have focused on improvement of morphological and physiological traits such as flower color, double flower phenotype, inflorescence length, and fertility (Sima 2019a, b; Daozong et al. 2018).

There are single and double flowered *M. incana* cultivars, and latter is more widely used for floriculture because it has more beautiful flowers. Seed production is only possible in single flowered individuals. It is impossible in double flowered plants due to degenerative pistils and stamens.

Therefore many researchers and breeders have aimed to produce of more double flowered plants than single flowered ones in *M. incana* and to develop markers to select them.

To date, several morphological traits such as cotyledon shape, serrate leaf, seed color, and leaf color have been used in early selection of double flowered stock individuals, because these traits were linked to double flowers (Saunders 1911; 1915; 1921; 1928; Ecker et al. 1993).

Takashi and Kanae (2018) identified the gene related to double flowers and developed a molecular marker to select double flowered plants efficiently, based on the study of double flower-linked molecular markers in Japanese gentian (Tasaki et al. 2017).

In general, the double flower trait is inherited by a single locus, *s* and it is linked to leaf color. In some stock cultivars, leaf color is a simple morphological marker, that is, green color indicates single flowered individual while pale green color represents double flowered individual (Ecker et al. 1993).

And also the fertility of plant is an important trait in cultivation and breeding of stock as well as other plants. Consequently, it is necessary to make a good stock cultivar with high fertility, different colors, and being capable of early discrimination of double flowered phenotype, in culture and production for commercial use.

Random amplified polymorphic DNA technique has been widely applied to evaluate genetic diversity on intraspecific level such as Ocimum (*Lamiaceae*) (Tanmay et al. 2016) and Swertia (*Gentianaceae*) (Prabhjot et al. 2019) and interspecific level including *Citrullus colocynthis* (Kumar et al. 2017), *Lactuca sativa* L. (Shubhangi et al. 2018), *Nilgirianthus ciliates*, *Artemisia herba*-alba (Khaled et al. 2019).

Moreover, RAPD method has also been used to assess genetic diversity on the level of variety or cultivar in following plants, for example, common beans (*Phaseolus vulgaris* L.) (Maciel et al. 2001), ginger (Siddharth et al. 2007), apricot (*Prunus armeniaca* L.) (Chroboková et al. 2011), cowpea (Masvodza et al. 2014), *Prosopis cineraria* (L.) Druce (Palaiyur et al. 2016), flax (*Linumu sitatissimum* L.) (Rozhmina et al. 2016), and *Penthorum chinense* Pursh (Zhiqiang et al. 2017).

And also ISSR method has been widely used in genetic diversity analysis because of its high reproducibility than RAPD method (Domenyuk et al. 2002; Galvan et al. 2003; Dogan et al. 2007).

However, in stock (*M. incana*), there are few reports on the study using molecular markers (Amaal 2009; Bekir et al. 2016; Takashi and Kanae 2018).

The present work aimed to: (1) make superior cultivars with different colors, high fertility, being capable of early selecting only double flowered seedlings by leaf color and examine morphological characteristics and (2) select RAPD and ISSR primers for cultivar certification and identification to culture and maintain good commercial stock cultivars.

The white cultivar is highly fertile, but it is not possible to distinguish between single flowered individuals and double flowered ones. Otherwise, in case of the pink cultivar, although it shows very low fertility, it is possible to select double flowered seedlings according to leaf color; single flower phenotype with green color and double flower phenotype with pale green color.

Here we evaluated genetic diversity among new double flowered stock cultivars obtained in cross “white” cultivar × “pink” cultivar by using morphological traits including leaf color, leaf shape, flower color and double flower phenotype, and RAPD and ISSR markers.

In this study we made some good stock cultivars with different colors including pink, pale pink and white (high fertile and capable of distinguishing between single and double flowered seedlings) through outcrossing between white cultivar (high fertile but impossible to select double flower phenotype) and pink

cultivar (vis. versa). In newly obtained stock cultivars, it is possible to distinguish between single and double flower seedlings by leaf color, having about 70 ~ 75% of fertility, thereby they are efficient good cultivars than both parent cultivars in seed production and culture.

Additionally, our cultivars were classified into two main groups, including the pink and the white cultivars respectively, the genetic distances among them varied from 0.03 to 0.24, with similarity to the previous research results in apricot and cowpea (Chroboková et al. 2011; Masvodza et al. 2014).

In the future, by using our selected RAPD and ISSR primers, the present method might be contributed to establish the cultivar certification, identification, and conservation system in culture and management of double flowered stock cultivars with high fertility and different colors.

Materials And Methods

Plant material and cross

Matthiola incana R. Br. cultivar “white” (DPR Korea) as a maternal parent and “pink” (DPR Korea) as a paternal parent were grown in a greenhouse at Pyongyang floriculture institute. In the cultivar “white”, plant height is high with no hairy leaves, white flowers, and high ratio of seed production (approximately 95%) otherwise, in the cultivar “pink”, plant height is lower than “white” with hairy leaves, pink flowers, and very low ratio of seed production (about 2.2%), besides with different leaf color between single and double flowered individuals.

We fertilized a “white” plant’s ovules using pollen produced by “pink” plant.

We obtained F_1 offspring by growing 320 seeds resulted from a hybridized plant, and mixed the seeds by self-fertilizing F_1 heterozygous plants. In the F_2 progeny produced from 10 plants of them, the selection was performed according to floral and leaf’s traits among 1757 plants. Subsequently, we conducted a family selection in the F_3 generation and selection of fixed line in the F_4 generation, finally obtained 4 pink, 2 pale pink and 2 white stock cultivars fixed in targeting traits in the F_5 generation.

Morphological analysis

Observations were made on each plant for phenotypic traits such as leaf color, leaf shape and flower color.

The following plant measurements were recorded: plant height, stem length, number of leaves per plant, leaf length and width, flower diameter, inflorescence length; numbers of flowers in inflorescence and pod (silique) length.

The data obtained from the research study was statistically analyzed by using ANOVA in Statistica ver. 6.0 and means were compared using Tukey’ s tests at the 5% level of significance. All means are presented with standard error.

Molecular analysis

Genomic DNA isolation

Fresh leaf samples were collected from individuals of all cultivars according to flower type (single or double) and leaf shape (hairy and no hairy). Genomic DNA were isolated from 500 mg of fresh leaf material using CTAB method (Cetyl Trimethyl Ammonium Bromide) developed by Doyle and Doyle (1990).

Qualitative and Quantitative estimation of DNA

DNA quality was assessed by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm providing a value of 1.8 which determines pure DNA preparation. Quality of DNA fragment was electrophoretically analyzed through 0.8% agarose gel using 1X TAE buffer at 50 V for 45 mins.

RAPD PCR amplification

RAPD PCR amplification was performed to amplify randomly unknown target sequences by using arbitary random primers according to the protocol described by Williams et al. (1990). PCR was carried out in a 25µl reaction volume containing 2.5µl of 10X Taq buffer, 2.5µl of dNTPs, 2.5µL of $MgCl_2$, 1µl of primer, 0.1µL 0.1U/µL *Taq* polymerase, 1µl of temple DNA (100 ng/ µl) and 15.4 µl of ddH_2O for each sample in a Mastercycler (nexus gradient).

We initially tested 25 primers from RAPD primer set (Opéron, sets D, H, I, N and P) on two individuals for each of the eight cultivars. We selected 10 primers (OPD3, OPH18, OPI14, OPN4, OPP19, OPH8, OPH19, OPP9, OPP17 and OPP20) with both reproducible and polymorphic variation with well defined and darkly staining bands.

Mastercycler (nexus gradient) programmed for an initial denaturation step of 94°C for 5 min, followed by 40 cycles of 30 s at 94°C, 60 s at 37°C and 90 s at 72°C, a final extension step of 72°C for 2 min.

Amplification products were separated on 1.5% agarose gels in 1×TAE buffer at 80 V for 1h 30 mins.

Gels were stained with ethidium bromide and photographed under UV light by using Geldoc-It™ (USA).

ISSR PCR amplification

ISSR PCR amplification was performed by using 7 primers with high GC content according to the protocol described by Bekir et al. (2016).

Data analysis

Only clear and polymorphic DNA bands were used for data analysis. The bands were scored as present (1) or absent (0) and a binary data matrix was constructed. DNA fragments of identical size amplified with the same primer were considered to be the same DNA marker.

To examine the genetic relationship among populations, a dendrogram was constructed using the unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) and principal coordinate analysis (PCA) was performed with NTSYSpc version 2.20 (Rohlf 2008).

Based on RAPD and ISSR data, the POPGENE ver. 1.32 (Yeh et al. 2000) was used to estimate values meaning genetic diversity: number of polymorphic band, observed number of alleles, effective number of alleles (Kimura and Crow 1964), Nei's (1973) gene diversity (h), Shannon's information index (I) (Lewontin 1972) and genetic distances among populations (Nei 1978).

A dendrogram of the populations was constructed which has been based on the unweighed pair group method (UPGMA) analysis of Nei's (1978) genetic distances. And also, G_{ST} representing population differentiation and gene flow (Nm) among populations were estimated.

Using the Arlequin ver. 3.5.2.2 (Excoffier and Lischer 2010), an analysis of molecular variance (AMOVA) was performed to evaluate the distribution of genetic variation within and among stock cultivars, and the Fixation index (Fst) was estimated too.

Results

Morpological diversity of new double flowered stock cultivars

We compared and analysed the morphological traits in new double flowered stock cultivars obtained from outcrossing including 4 pink, 2 pale pink and 2 white cultivars (Fig. 1).

Table 1
Characteristics of morphological traits in stock cultivas

No	cultivar	percent of double flowered plants /%	Flower color	double flower phenotype	Population ID	Flower diameter /cm	inflorescence length/cm	number of flowers in inflorescence	Leaf color	Leaf shape	Plant height /cm	number of leaves per plant	Leaf leng /cm
1	□pink□ (♀)	54.3 ± 5.8	pink	double	1	5.0 ± 0.5	20.5 ± 2.8	16.5 ± 2.4	pale green	no hairy	50.8 ± 2.4	17.5 ± 2.4	12.8 ± 1.4
				single	2	3.6 ± 0.3	15.3 ± 2.5	15.3 ± 1.8	green		46.1 ± 1.8	18.5 ± 2.1	12.1 ± 1.8
2	□white□	53.4 ± 5.6	white	double	3	5.2 ± 0.7	27.5 ± 1.9	21.0 ± 1.5	pale green	no hairy	80.8 ± 3.4	27.7 ± 0.9	15.4 ± 2.2
				single	4	4.0 ± 1.1	17.6 ± 2.2	12.4 ± 0.9	green		79.8 ± 5.9	28.9 ± 1.6	12.1 ± 2.3
3	□white□ (♂)	47.4 ± 4.4	white	double		4.7 ± 0.7	19.8 ± 2.6	18.3 ± 1.1	green	hairy	53.8 ± 6.2	26.2 ± 2.3	12.4 ± 1.9
				single	5	3.6 ± 0.4	18.3 ± 3.3	17.3 ± 1.3	green		50.7 ± 3.8	28.8 ± 2.1	11.4 ± 2.4
4	□pink 1□	49.3 ± 2.8	Pink	double	6	4.4 ± 0.7	28.8 ± 1.7	22.8 ± 2.4	pale green	no hairy	80.8 ± 7.6	27.7 ± 2.7	15.4 ± 1.9
				single	7	3.6 ± 0.2	18.9 ± 2.3	16.5 ± 1.7	green		79.8 ± 4.5	28.9 ± 1.1	12.1 ± 2.4
5	□pink 2□	52.8 ± 3.6	Pink	double	8	4.7 ± 0.8	27.9 ± 3.4	22.4 ± 2.7	pale green	hairy	80.8 ± 6.3	27.7 ± 2.5	15.4 ± 2.3
				single	9	4.0 ± 0.4	17.7 ± 2.9	16.2 ± 1.9	green		79.8 ± 4.2	28.9 ± 1.8	12.1 ± 1.2
6	□pink 3□	53.2 ± 3.8	Pink	double	10	5.2 ± 0.9	28.2 ± 2.4	21.0 ± 2.6	pale green	no hairy	80.8 ± 5.3	27.7 ± 3.2	15.4 ± 2.4
				single	11	4.0 ± 0.3	18.5 ± 3.2	15.4 ± 1.7	green		79.8 ± 2.9	28.9 ± 2.9	12.1 ± 2.9
7	□pink 4□	46.4 ± 2.7	Pink	double	12	5.2 ± 0.5	27.3 ± 2.6	22.0 ± 2.6	pale green	hairy	80.8 ± 3.4	27.7 ± 2.2	15.4 ± 1.8
				single	13	4.0 ± 0.2	17.8 ± 2.1	14.8 ± 1.7	green		79.8 ± 2.8	28.9 ± 1.9	12.1 ± 1.3
8	□pale pink 1□	51.7 ± 4.2	Pink	double	14	5.0 ± 0.4	20.9 ± 3.8	18.3 ± 2.4	pale green	no hairy	45.4 ± 3.6	26.8 ± 2.0	12.2 ± 1.1
				single	15	3.6 ± 0.7	17.8 ± 2.7	17.3 ± 1.7	green		47.8 ± 3.8	24.8 ± 2.3	11.2 ± 1.4
9	□pale pink 2□	49.4 ± 2.8	Pink	double	16	4.4 ± 0.6	19.9 ± 1.2	18.3 ± 2.1	pale green	hairy	44.4 ± 4.2	15.5 ± 2.8	12.6 ± 1.9
				single	17	3.6 ± 0.8	15.9 ± 2.4	17.3 ± 1.6	green		43.8 ± 3.9	16.5 ± 2.6	12.4 ± 1.6
10	□white 1□	48.4 ± 4.6	White	double	18	4.7 ± 0.3	28.5 ± 2.1	20.8 ± 1.4	pale green	no hairy	80.8 ± 2.8	27.7 ± 3.1	15.4 ± 2.8
				single	19	4.0 ± 0.4	18.1 ± 1.7	14.8 ± 2.3	green		79.8 ± 4.2	28.9 ± 1.9	12.1 ± 0.4
11	□white 2□	53.2 ± 4.1	White	double	20	5.2 ± 0.5	27.9 ± 2.9	20.5 ± 2.0	pale green	no hairy	80.8 ± 3.9	27.7 ± 2.3	15.4 ± 1.7
				single	21	4.0 ± 0.9	18.0 ± 1.9	14.4 ± 1.6	green		79.8 ± 2.7	28.9 ± 3.1	12.1 ± 1.6

As shown in Table 1, in newly obtained 8 stock cultivars, the ratio of double flowered plants was about 50%, and some traits such as flower diameter, leaf length and leaf width, showed no significant differences ($P > 0.05$) in all 11 cultivars including both parent. Conversely, in other traits including inflorescence length, number of flowers per inflorescence, plant height, number of leaves per plant, “white” cultivar was markedly bigger than “pink” cultivar ($P < 0.05$), newly obtained pink and white cultivars were not significantly different from maternal parent ($P > 0.05$), “white”, whereas pale pink cultivars were not significantly different from paternal parent, “pink” ($P > 0.05$). Besides, in pod length and the percent of fertile plants, new eight cultivars had medium values between maternal and paternal parent cultivars, not showing a significant difference ($P > 0.05$) according to cultivar.

Molecular identification of new stock cultivars using RAPD and ISSR markers

Genetic relationship among twenty one stock individuals

In the present study the genetic relationship among and within cultivars of *M. incana* were analysed by ten informative RAPD and ISSR primers (Table 2).

Table 2
Primers used for RAPD and ISSR analysis, total number of scored fragments and their size range

Primer	Sequence (5′→ 3′)	Fragment size range (bp)	Total number of bands
OPD3	5′ GTCGCCGTCA 3′	100–4000	10
OPH18	5′ GAATCGGCCA 3′	300–2000	6
OPI14	5′ TGACGGCGGT 3′	100–2500	11
OPN4	5′ GACCGACCCA 3′	100–3000	12
OPP19	5′ GGGAAGGCA 3′	200–2000	5
ISSRM1	$(AGC)^6$-G	200–3000	10
ISSRM2	$(ACC)^6$-G	150–2000	10
ISSRM3	$(AGC)^6$-C	100–4000	12
ISSRM5	$(GA)^9$-C	200–3000	11
ISSRF3	$(AG)^8$-CG	150–2000	10

Based on above obtained RAPD and ISSR profiles (Fig. 3), genetic relationship among 21 stock samples was estimated using NTSYSpc2.11.

We obtained data matrix based on Nei (1973)'s genetic distance and constructed a dendrogram using UPGMA method.

As shown in Fig. 4, at genetic distance of 0.15, dendrogram grouped all individuals in two clusters, and principal coordinate analysis result (Fig. 5) was agreed with cluster analysis (Fig. 4).

First cluster, □ contained No. 1, 9, 10, 12, 6, 14, 16 individuals, being pink and single flowered. Second cluster, □ contained No. 2, 13, 8, 11, 7, 15, 17 individuals, being pink and double flowered. Third cluster, □ contained No. 3, 4, 18, 20 individuals, being white and single flowered. Final fourth cluster, □ contained No. 5, 21, 19 individuals, being white and double flowered. This suggests that our selected RAPD and ISSR primers might reflect not only flower color but also double flower phenotype.

Genetic relationship among eleven stock cultivars

Genetic relationship among eleven stock cultivars distinguished by flower color and leaf shape was evaluated using PopGene.version 1.31.

Table 3
Nei's (1978) genetic distance among eleven studied stock populations. The abbreviated population names are given according to Table 1.

pop ID	1	2	3	4	5	6	7	8	9	10	11
1	****	0.8741	0.8612	0.9480	0.9694	0.9604	0.9636	0.9440	0.9393	0.8437	0.8638
2	0.1346	****	0.9439	0.8031	0.8296	0.8219	0.8282	0.8124	0.8152	0.8826	0.8918
3	0.1494	0.0577	****	0.8280	0.8150	0.8169	0.8132	0.7925	0.8066	0.9424	0.9514
4	0.0534	0.2193	0.1888	****	0.9153	0.9163	0.9144	0.8845	0.8964	0.8055	0.8501
5	0.0311	0.1868	0.2015	0.0885	****	0.9549	0.9597	0.9209	0.9393	0.7928	0.8044
6	0.0404	0.1961	0.2022	0.0874	0.0461	****	0.9507	0.9124	0.9169	0.7855	0.7969
7	0.0371	0.1885	0.2068	0.0895	0.0412	0.0505	****	0.9104	0.9048	0.7947	0.8160
8	0.0576	0.2077	0.2326	0.1227	0.0824	0.0917	0.0939	****	0.9681	0.8112	0.8092
9	0.0626	0.2044	0.2149	0.1094	0.0626	0.0867	0.1000	0.0324	****	0.8074	0.8155
10	0.1699	0.1249	0.0594	0.2163	0.2322	0.2415	0.2298	0.2092	0.2140	****	0.9684
11	0.1464	0.1146	0.0498	0.1624	0.2177	0.2270	0.2033	0.2118	0.2040	0.0321	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

As shown in Fig. 6, eleven stock cultivars were classified into two main groups; pink and white cultiivar group.

The genetic distance values for all 11 cultivars ranged from 0.03 to 0.25 showing the lowest genetic distance (0.02415) between “pink 3” and “white 1”, while the highest genetic distance (0.0321) between “white 1” and “white 2” (Table 3).

In the group of white cultivars, new cultivar “white 2” was genetically closest to control cultivar “white” (genetic distance- 0.0498), and new cultivar “white 1” was the most distant to maternal cultivar “white” (genetic distance- 0.1062). Otherwise, in the group of pink cultivars, new cultivar “pink 2” was the genetically closest to paternal cultivar “pink” (genetic distance- 0.0534), and new cultivar “pink 1” was the most distant to new cultivar “pale pink 1” (genetic distance- 0.0965).

Among 11 newly obtained stock cultivars, differentiation index ($G_{ST)}$ and gene flow index (Nm) were 0.639(0.1309-1.000) and 0.2817(0.0430–3.3195) respectively.

The values of genetic diversity indices for studied stock cultivars are given in Table 4. The highest n_a was observed for “pale pink 2” (1.26) and the least for “pink 3” (1.07), showing the lowest Shannon’s index (0.04) and Nei’s gene diversity (0.03) as well.

Nei’s gene diversity were estimated to be 0.03 and 0.11 and the Shannon indices of the *M. incana* cultivars ranged from 0.04 to 0.16.

Table 4
Genetic diversity indices for eleven stock cultivars. N_{PB}- number of polymorphic bands, P_{PB}-percentage of polymorphic bands, n_a – observed number of alleles, n_e –effective number of alleles, h - Nei’s (1987) gene diversity, I - Shannon’s information index

Cultivar	N_{PB}	P_{PB}	n_a	n_e	h	I
pink (paternal)	5	10.87	1.11 ± 0.31	1.08 ± 0.22	0.05	0.07
white	0	0	1	1	0	0
white (maternal)	8	17.39	1.17 ± 0.38	1.23 ± 0.27	0.07	0.11
pink 1	8	17.39	1.17 ± 0.38	1.23 ± 0.27	0.07	0.11
pink 2	5	10.87	1.11 ± 0.31	1.08 ± 0.22	0.05	0.07
pink 3	3	6.52	1.07 ± 0.25	1.05 ± 0.18	0.03	0.04
pink 4	7	15.22	1.15 ± 0.36	1.11 ± 0.26	0.06	0.09
pale pink 1	8	17.39	1.17 ± 0.38	1.23 ± 0.27	0.07	0.11
pale pink 2	12	26.09	1.26 ± 0.44	1.18 ± 0.31	0.11	0.16
white 1	11	23.91	1.24 ± 0.43	1.17 ± 0.31	0.10	0.14
white 2	7	15.22	1.15 ± 0.36	1.11 ± 0.26	0.06	0.09

Table 5
Analysis of molecular variance (AMOVA) based on RAPD profiless in eleven stock cultivars

Source of variation	degrees of freedom	Sum of squares	Variance components	Percentage of variation (%)	P
Among cultivars	10	283.562	2.584	67.27	< 0.0001
Within cultivars	20	105.600	1.257	32.73	< 0.0001
Total	30	389.162	3.841		

Genetic variation among cultivars was more than that within cultivars, as indicated by the sum of squares values calculated by AMOVA using Arlequin ver. 3.5.2.2 (Table 5). Of the total genetic diversity, 67.27 % was attributable to differences among culivars and 32.73% was to differences within culivars, showing a significant varietal differentiation in newly obtained stock cultivars. The fixation index (Fst) was 0.673.

Discussion

Because double flowered cultivars have superior ornamental value to single flowered ones, stock breeders have attempted to make double flowered cultivars with different colors.

Additionally, in breeding of *M. incana*, the studies also have been reported to identify the morphological and molecular markers which could be used to discriminate the double flowered individuals.

Ecker et al. (1993) founded that the *c* allele for sinuate leaf shape might be recessive to the *C* allele for normal entire leaf and it was tightly linked to the *s* recessive allele for double flowering, suggesting its role as a double flowering marker in the cultivation and the breeding of *M. incana.*

Takashi and Kanae (2018) revealed that co-dominant marker, *MiAG* could be used for early discrimination of double flowered individuals among seedlings not showing phenotypic differences. He examined that the single flowered individuals of cultivars being distinguished double flowered ones according to

cotyledon shape ('Kiss me white', 'Iron white', 'Quartet cherry'), serrated leaf ('Pygmy white'), seed color ('White wonder no.2') were heterozygotes *MiAG/miag1*, whereas the individuals correlated with leaf color (in 'Aida' and 'Opera') were heterozygotes *MiAG/miag2.*

In this study we made the garden stock varieties with different colors (pink, pale pink and white) and high fertility which were capable of discriminating double flowered individuals in cross "white" cultivar (high fertile but impossible in selection of double flowered plants) × "pink" cultivar (very low fertile but possible in selection of double flowered plants). Considering with Takashi and Kanae et al. (2018)'s result, our newly raised stock varieties are likely *MiAG/miag2* heterozygotes being capable of selecting double flowered ones among the seedlings according to leaf color (Fig. 2), and harboring high fertility, therefore they might be superior to both parents cultivars in the cultivation and seed production for commercial use.

To date, random amplified polymorphic DNA (RAPD) method have been widely applied to assess the intra- and inter-specific genetic diversity in many plants.

Tanmay et al. (2016) described the diversity of nine genotypes in the genus *Ocimum* from India based on RAPD markers combining with morphological and chemical markers.

Prabhjot et al. (2019) assessed the molecular diversity among 48 accessions of five *Swertia* species collected from Western Himalayas, India using 18 RAPD markers.

RAPD primer based genetic diversity within the species were reported earlier in several plant species such as *Citrullus colocynthis* (Kumar et al. 2017), lettuce (*Lactuca sativa* L.) (Shubhangi et al. 2018), *Nilgirianthus ciliates* (Ramakrishnan et al. 2019) and *Artemisia herba*-alba (Khaled et al. 2019).

Besides, RAPD technique has also been used to examine the genetic diversity on the level of variety or cultivar (Maciel et al. 2001; Siddharth et al. 2007; Chroboková et al. 2011; Masvodza et al. 2014; Palaiyur et al. 2016; Rozhmina et al. 2016; Zhiqiang et al. 2017).

Palaiyur et al. (2016) showed that the similarity coefficients ranged from 0.57 to 0.78 by using nine of 80 RAPD primers and they could be applied to identify different varieties in *Prosopis cineraria* (L.) Druce.

Rozhmina et al. (2016) revealed genetic similarity between cultivars of fiber flax from Russia and other the European countries using RAPD markers. On the other hand, ISSR method is more reproducible than RAPD method, so it is more widely used in the analysis of genetic diversity in many plants such as maize (Domenyuk et al. 2002), common bean (Galvan et al. 2003) and *Jurinea* (Dogan et al. 2007).

And also, recent researchers (Ramakrishnan et al. 2019; Joyashree et al. 2019; Zhiqiang et al. 2017; Kumar et al. 2017) reported that RAPD and ISSR markers could be applied together to reveal genetic relationship between germplasm lines and to establish the cultivar identification system using cluster analysis and PCA.

However, there are few reports in stock plant (*Matthiola incana*) to analyze genetic relationship using molecular markers.

Amaal (2009) performed molecular systematic analysis for twenty two taxa belonging to *Brassicaceae* including *Matthiola incana* and *Matthiola longipetala* subsp. *livida* using RAPD markers and suggested its role for varietal identification and assessment of genetic relationships even between closely related species.

And Bekir et al. (2016) studied phylogenetic relationship of Turkish species of *Matthiola* based on only ISSR markers.

In contrast to previous study, the present method might be applied to identify new double flowered stock plant varieties obtained through outcrossing, by using RAPD and ISSR markers together.

By combining new selected RAPD and ISSR primers, our analysis method grouped stock plant varieties into two main clusters, one including pink varieties and the other including white ones. Besides single and double flowered individuals were classified in each cluster, and RAPD based genetic distances varied from 0.03 to 0.24 among these varieties which is similar to Chroboková et al. (2011) (0.02 ~ 0.45), Masvodza et al. (2014) (0.1 ~ 0.22)' s research result and has somewhat smaller scale than Palaiyur et al. (2016) (0.22 ~ 0.43), Maciel et al. (2001) (0.2 ~ 0.75), and Siddharth et al. (2007) (0.34 ~ 0.74)' report.

Conclusion

We bred new double flowered stock cultivars with different colors and high fertility in cross white cultivar that was high fertile but impossible to select double flower phenotype × pink cultivar (vice. versa). RAPD and ISSR markers selected in this study can be used to identify different stock cultivars according to flower color and flowered phenotype, therefore in addition to morphological traits, they will be applied to cultivar certification, identification, and establishing conservation system in their culture and management.

Abbreviations

RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat)

Declarations

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank all horticulturists of Pyongyang floriculture institute who helped culturation during stock breeding.

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Figures

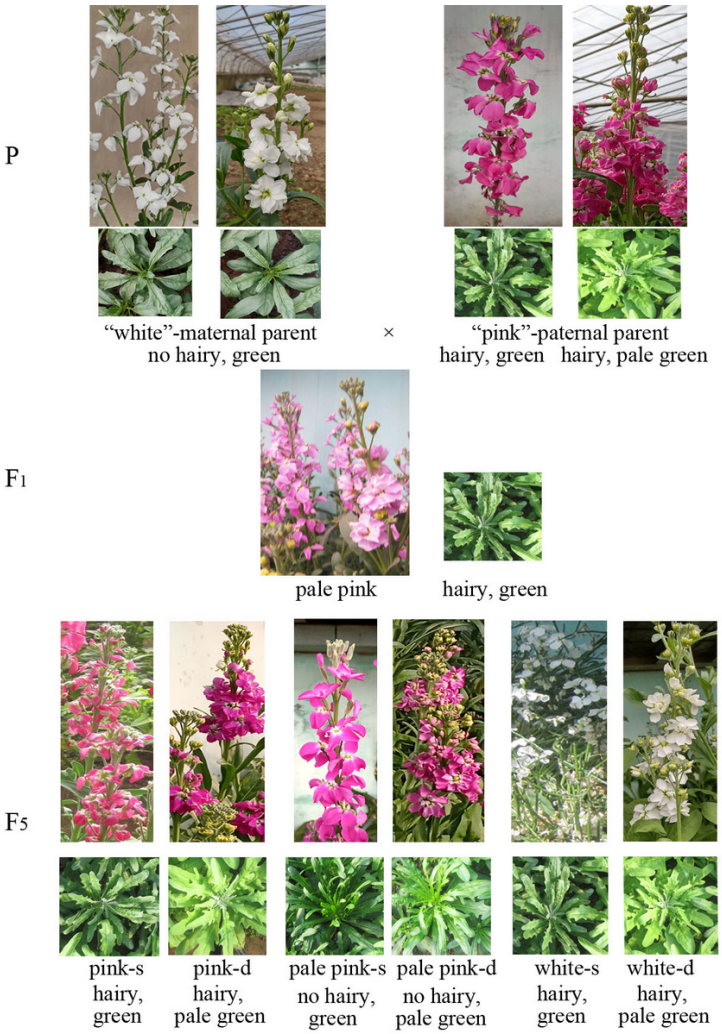

Figure 1

Phenotypes of newly obtained stock plants and both parent plants "-s" means single flowered phenotype and "-d" means double flowered phenotype.

Figure 2

Leaf color in young seedlings in newly obtained stock plants (green: double flowered seeding, pale green: single flowered seeding)

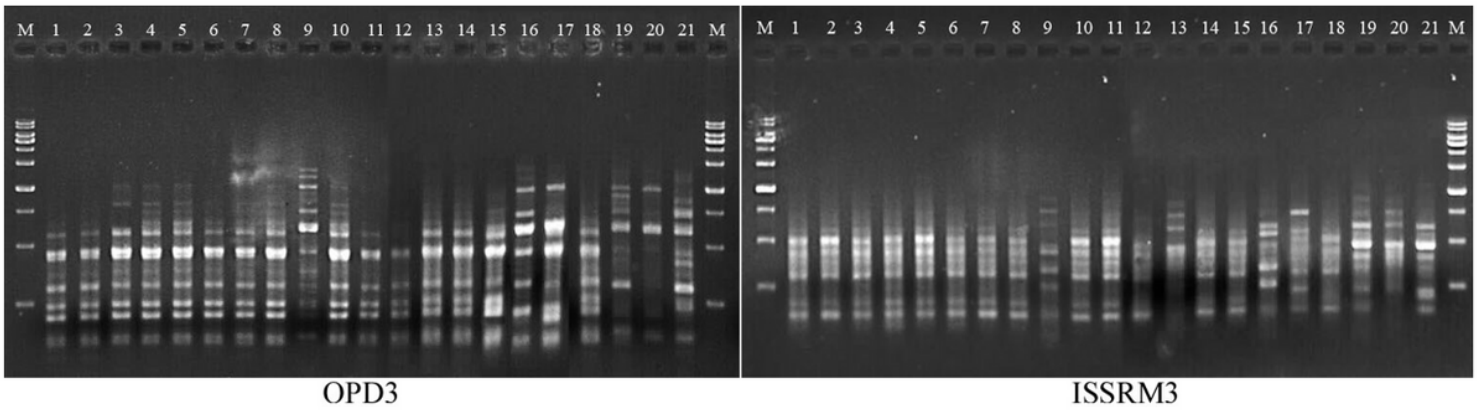

Figure 3

RAPD and ISSR agarose gel electrophoresis profiles of eleven stock cultivars using OPD3(left) and ISSRM3(right) primers. Lines marked by numbers (1, 2, 3..., 21) represent the individuals that belong to cultivars listed in table 1. M –1 kb ladder

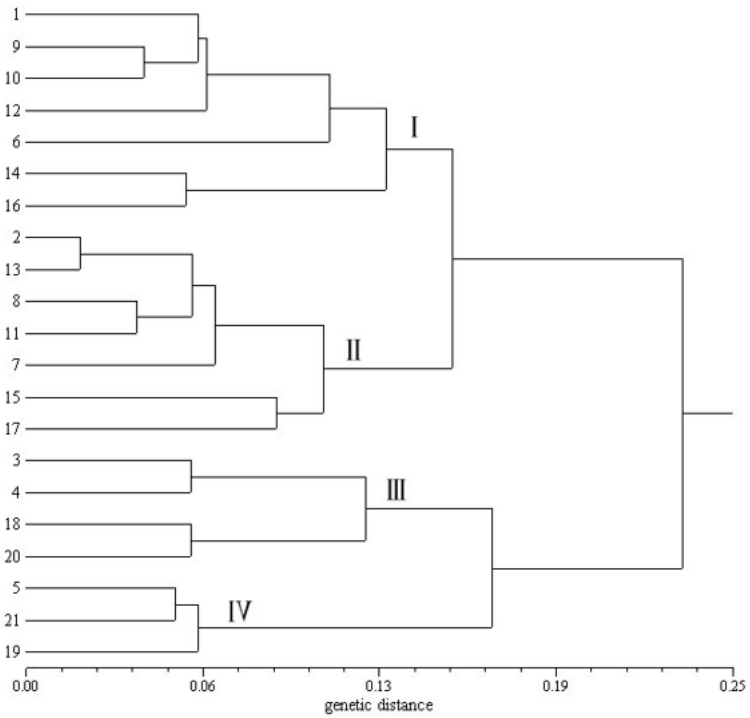

Figure 4

UPGMA dendrogram illustrating the genetic relationships between M. incana genotypes based on Nei's genetic distance. Individual Id is as population Id in table 1. 1,2 are male parents, 5 is the female parent.

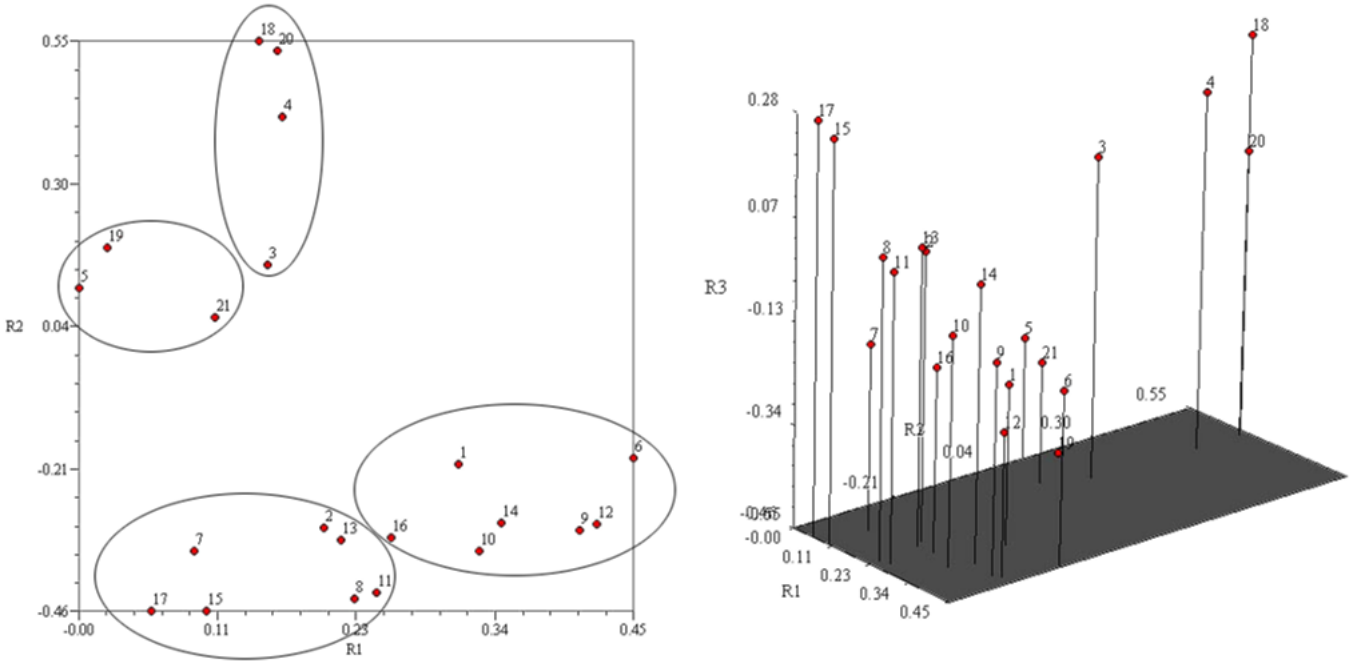

Figure 5

2-D(left) and 3-D(right) PCA plot based on RAPD and ISSR data in M. incana genotypes.

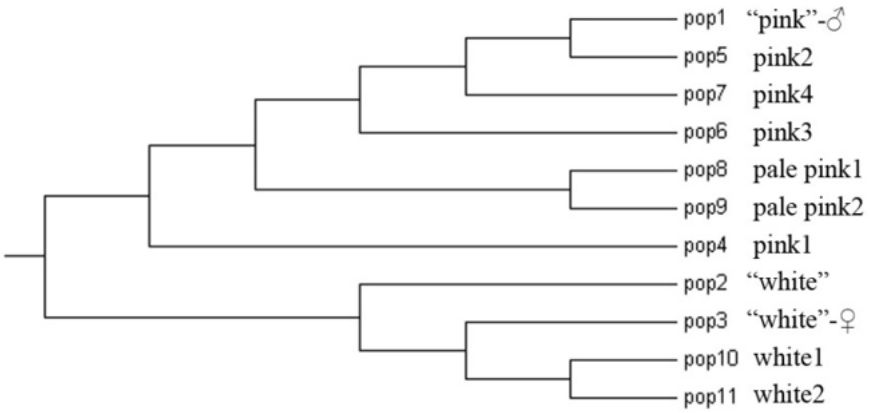

Figure 6

UPGMA dendrogram illustrating the genetic relationships between eleven cultivars of M. incana.