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Study of genetic diversity in local rose varieties (*Rosa* spp.) using molecular markers

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Abstract. This study was undertaken to evaluate genetic diversity in a germplasm consisting of rose varieties. Genetic distances were estimated using three different molecular marker techniques including: start codon targeted (SCoT), conserved DNA–Derived Polymorphism (CDDP) and directly amplified minisatellite DNA (DAMD). According to the results, the average polymorphism information content was 0.37, 0.36, and 0.36 for SCoT, CDDP and DAMD markers, respectively indicating that the studied marker types were equal in terms of assessing diversity. Cluster analysis using SCoT and CDDP divided the varieties to four distinct clusters whereas DAMD markers data, grouped the varieties into three clusters. There was a positive significant correlation ($r=0.92$, $p<0.01$) between similarity matrix obtained by SCoT and CDDP. Results suggested that the efficiency of SCOT, CDDP and DAMD markers had a relatively same efficiency in fingerprinting of varieties. This is the first time that the efficiency of the three molecular markers have been compared with each other in a set of rose samples. The results showed that the studied markers had an appropriate polymorphism and thus were suitable for the study of genetic diversity in rose.

Keyword: Fingerprinting, PIC, molecular marker, genetic distance, correlation.

Introduction

Rose (*Rosa sp.*) is one of the world's most important commercial flowers which are used as garden plant, cut flower and source of essential oil [GUDIN 2000]. Rose breeding programs are mainly based on the production of new hybrids and evaluation of genetic diversity is an essential tool for such programs.

Molecular markers are of the methods used in the study of genetic diversity both within and between species [POWELL *et al*., 1996].

Amongst them, DNA markers are the most important and useful marker systems which are widely used.

Previously, genetic variation has been assessed in rose genotypes using some molecular markers such as SSR [ZHANG *et al.*, 2013; SAMIEI *et al*., 2009], RAPD [JAN and BYRNE, 1999; AZEEM *et al*., 2012], and RFLPs [HUBBARD *et al*., 1992]. However, these markers have some weaknesses.

For example: need to high–quality DNA, laborious, complex to automate, need to radioactive labeling and characterization of probe are of great disadvantages of RFLPs.

Also, SSR marker system requires sequence information and may not be suitable across species. Similarly, disadvantages of RAPD markers include dominant, non–reproducibility and lack of detection of allelic system [MIAH *et al*., 2013].

In recent years, new markers have been developed which can be considered as suitable alternatives for previous markers [GUPTA and RUSTGI 2004].

These new markers such as CDDP [COLLARD and MACKILL 2009a], SCoT [COLLARD and MACKILL 2009b] and DAMD [JEFFREYS *et al*., 1985] have been developed based on the conserved regions of genes and can easily generate functional markers related to a given plant phenotype [POCZAI *et al*., 2013].

Molecular markers that are extended in genome across different plant species, such as SCoT and CDDP, and have longer primers with higher annealing temperature will be more trustworthy and reproducible than arbitrary markers such as RAPDs or ISSR.

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SCoT and CDDP markers have been used in many crop plants such as wheat [HAMIDI *et al*., 2014], and chickpea [HAJIBARAT *et al*., 2015].

Here, we assessed genetic diversity in rose varieties using SCoT, DAMD and CDDP markers for first time.

Comparison between the above markers in estimating genetic relations among rose varieties was the other goal of this study.

Material and methods

Plant material. A total of 20 local rose varieties provided from the National Institute of Ornamental Plants (NIOP), Mahallat, Iran were studied (Table 1).

Genomic DNA extraction and DAMD marker analysis. DNA was extracted from 1 g of fresh leaves collected from 14–day–old seedling using the modified CTAB method [LASSNER *et al*., 1989] with the modification described by Torres and collab. [TORRES *et al*., 1993].

Table 1.

Names of local rose varieties evaluated in this research.

Name	Entry no.	Name	Entry no.
Vandenta	1	Musk rose	11
cool water	2	PV6	12
Rosa moyesii	3	Mo1	13
meinyature	4	Rose sp. ablgh	14
red s. meinyature	5	Black	15
Ablagh meinyature	6	PO1	16
R.vandenta	7	PY3	17
red meiyature	8	Rosa sp color	18
Marociya	9	P.R. vandenta	19
R.marociya	10	PY52	20

To produce DNA fingerprint profiles, ten primers were screened of which eight were selected based on GC content of 50–60 % and an annealing temperature of 49 °C (Table 2).

PCR amplification was performed in 25 µL reaction containing 1× PCR buffer, 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5–2.5 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase.

Table 2.

Primers used in DAMD, SCoT, and CDDP marker systems for study of genetic variation among 20 local rose variteies.

Marker system	Primer	Sequence 3′ to 5′	GC (%)	Annealing temperature
DAMD	UPR–2F	GTGT GC GA TC AG TT GC TG GG	60	49
	UPR–4R	AGGA CT CG AT AA CA GG CT CC	55	49
	UPR–6R	GGCA AG CT GG TG GG AG GT AC	65	49
	UPR–13R	TACA TC GC AA GT GA CA CA GG	50	49
	UPR–17R	AATG TG GG CA AG CT GG TG GT	55	49
	UPR–25F	GATG TG TT CT TG GA GC CT GT	50	49
	UPR–30F	GGAC AA GA AG AG GA TG TG GA	50	49
	UPR–38F	AAGA GG CA TT CT AC CA CC AC	50	49
SCoT	SCOT–1	CAACAATGGCTACCACCA	50	48
	SCOT–2	CAACAATGGCTACCACCC	55	48
	SCOT–11	AAGCAATGGCTACCACCA	50	48
	SCOT–13	ACGACATGGCGACCATCG	61	48
	SCOT–22	TACATCGCAAGTGACACAGG	55	48
	SCOT–28	CCATGGCTACCACCGCCA	66	48
	SCOT–36	GCAACAATGGCTACCACC	55	48
CDDP	KNOX–1	AAGGGSAAGCTSCCSAAG	45	48
	KNOX–2	CACTGGTGGGAGCTSCAC	61	48
	KNOX–3	AAGCGSCACTGGAAGCC	55	48
	WRKY– 1R	GTGGTTGTGCTTGCC	50	48
	WRKY–2R	GCCCTCGTASGTSGT	45	48
	WRKY–3R	GCASGTGTGCTCGCC	55	48

All amplifications were carried out in an Eppendorf thermocycler as followed: 94 °C for 2 min, followed by 40 cycles of 45 s denaturation at 94 °C, 1 min

annealing at 57 °C, and 2 min. elongation at 72 °C. The obtained amplicons were run on 1.5 % agarose gel, stained with ethidium bromide.

SCoT and CDDP analysis

In this study, seven SCoT and six CDDP primers designed by Collard and Mackill [COLLARD and MACKILL 2009a, 2009b] were applied (Table 2). These primers were 18–mer and their GC content ranged between 50 and 72 %.

Sequences were scanned for short conserved amino acid regions with the low permutations of possible codons.

Up to three degenerate nucleotides were included in a single primer.

Since plant exons are typically guanine–cytosine (GC) rich, some degeneracies were incorporated into primers corresponding to the third nucleotide position of a codon (i.e., G or C in the primer sequence was designed as an "S").

Primers were 15 to 18 nucleotides in length and had >60 % GC content. PCR proliferation was used in 25 µL reaction containing 1× PCR buffer, 50 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 3 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase (Cinnagen, Iran).

Thermal cycling (Eppendorf) initiated with 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 2 min, and extension at 72 °C for 2 min.

A final elongation step of 8 min at 72 °C was added.

Amplified PCR products were separated by gel electrophoresis on 1.5 % agarose gels, stained with ethidium bromide.

Data analysis

Polymorphic alleles were scored as presence or absent (1/0). DARwin version 5.0 was applied for analyzing pairwise genetic distances and for making the distance matrix [PERRIER *et al.* 2003].

The produced genetic distance was used to calculate the frequency of dissimilarity and dendrogram analysis using the unweighted neighbor–joining method (UNJ) [GASCUEL, 1997]. The bootstrap analysis running 1000 replication was employed to determine a sampling variance of the genetic similarities calculated from the data sets gained of different marker systems [PERRIER *et al.*, 2003].

The Mantel test of importance [MANTEL, 1967] was also applied to compare each pair of similarity matrices created. Almost of all methods were performed by NTSYSpc version 2.0 [ROHLF, 1997].

Polymorphic information content (PIC) values were calculated for each primer according to the formula: PIC=1–S(Pij)2; where Pij is the frequency of the ith pattern showed by the jth primer aggregated across all patterns revealed by the primers [BOTSTEIN *et al.*, 1980].

Results and discussion

The results of the banding pattern of electrophoresis showed that the three markers could demonstrate the high level of diversity existing among the individuals consequently; the markers were functional for each of 20 local rose varieties (Figure 1). PCR–based molecular markers can play an important role in the analysis of genetic diversity in such species. Fingerprinting data obtained using DAMD; SCoT and CDDP markers were as below:

SCoT Analysis

The used Seven SCoT primers generated 47 bands which were polymorphic up to 98 %. The maximum and a minimum number of amplified bands with 11 and 2 bands belonged to SCOT–13 and SCoT22, respectively.

The polymorphism value was varied from 77 % to 100 %. PICs ranged from 0.45 to 0.25 for primers SCOT–36 and SCoT22, respectively. Marker Index (MI) ranged from 0 (SCoT23) to 8.066 (SCoT31).

Primer SCoT13 (4.82) had the highest MI value while; Primer SCoT 22 (0.5) had the lowest. Cluster analysis classified the varieties into three major groups (Figure 2). Cluster I, II and III each contained seven, four and seven varieties while, PY52 and red melyature were not included in any group.

CDDP Analysis

CDDP primers produced a total of 32 bands (Table 3). The average number of polymorphic bands was 5.33 per primer

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ranged from 2 (Knox2) to 10 (WRKY–1R, WRKY–2R). The polymorphism percentage was 100 % with an average of 100 % showing a high polymorphism level. The average value of PIC was 0.37 per locus ranged from 0.25 to 0.41 (Table 4). Neighbor–Net cluster analysis based on CDDP divided local rose varieties into four clusters (Figure 3).

Clusters I, II, III and IV contained seven, four, seven members. Of these, some clusters had a relatively similar grouping pattern with those obtained by means of SCoT and DAMD markers.

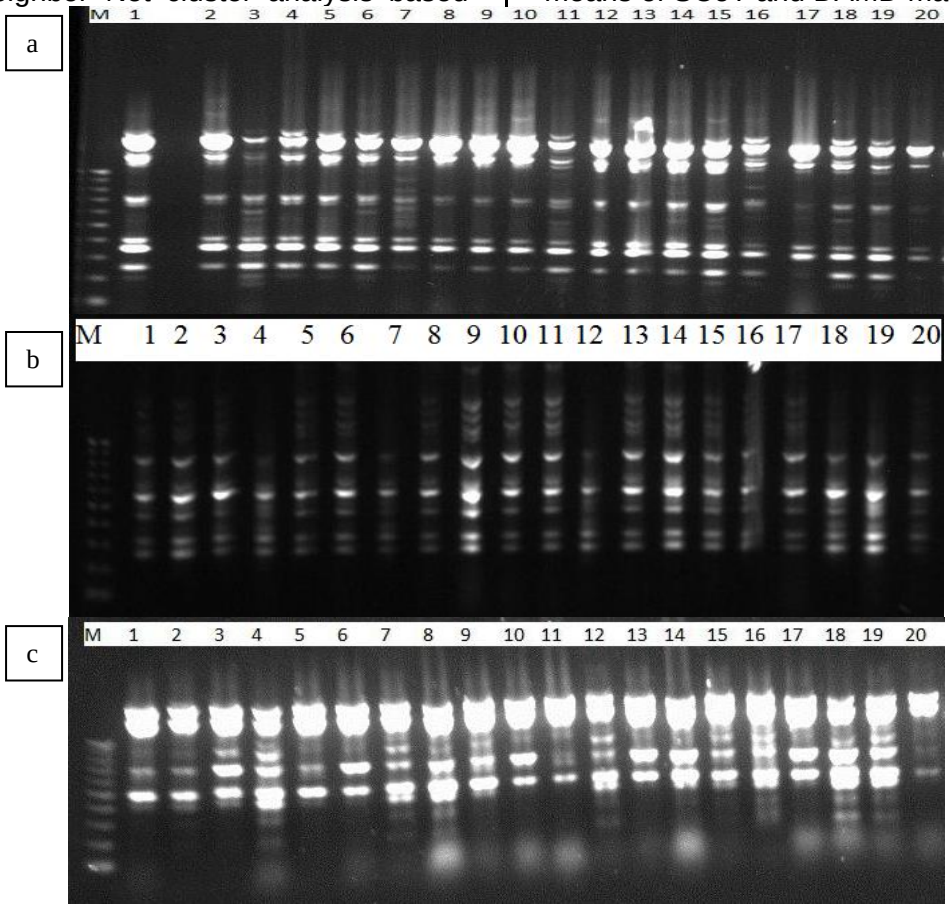

Figure 1. Amplification profile obtained with SCoT13 (a), URP13R (b) and WRKY–R1 (c) primers.

DAMD analysis

PCR amplification was successful for seven DAMD primer and produced 47 fragments (Table 3).

The number of proliferated alleles ranged from 4 to 9 with a mean of 5.8 polymorphic bands per primer.

Averagely, the PIC value was 0.37 per locus (0.26 to 0. 43) (Table 3).

Cluster analysis grouped the varieties into three distinct clusters (Figure 4).

Cluster I contained 11 local varieties and cluster II contained six local varieties. Cluster II revealed similar grouping pattern with those obtained by SCoT data.

Cluster III included three local varieties.

Correlation among marker systems

Estimated cophenetic correlation coefficient (CCC) indicated a good fit of data obtained by the three markers (DAMD=0.84; SCoT=0.83 and CDDP=0.89) representing consistent results.

The CCC was notably high (0.92 between SCoT and CDDP, 0.89 between DAMD and SCoT, and 0.85 between CDDP and DAMD, $P<0.01$) (Table 4) indicating a good relationship between genetic distances obtained through all marker techniques.

Also, high CCC between SCoT and CDDP indicated a similarity in DNA sequence variation at primer binding

regions between the two markers which is important in hybridization programs.

Comparison between the results derived from this study with those obtained using SSR [ZHANG *et al.*, 2013], and RAPD markers [JAN and Byrne, 1999] revealed that the three studied marker systems had a relatively higher polymorphism percentage and PIC values.

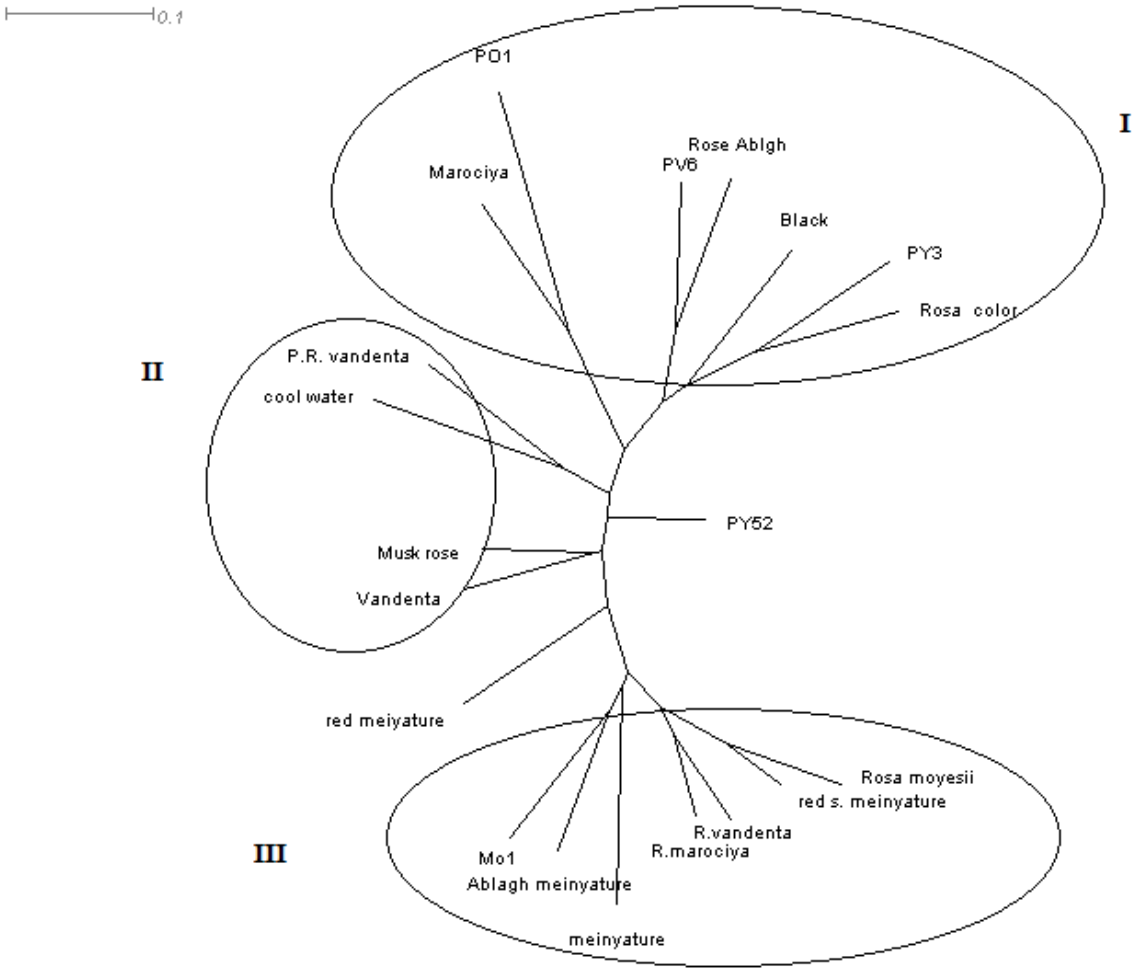

Figure 2. Dendrogram of the 20 local rose varieties using SCoT markers based on genetic distance.

Comparison among the studied markers

In the present study, the average amount of genetic distances obtained by CDDP, SCoT, and DAMD markers were 0.36, 0.37, and 0.36, respectively.

There was a relatively significant level of polymorphism within the varieties which was in agreement with findings of Saeed and collab. and Ghaffari and collab. but was not consistent with reports of Byrne, and Matsumoto and collab. who reported a low level of genetic diversity within rose germplasm [SAEED *et al.*, 2011, GHAFFARI *et al.*, 2014, BYRNE, 1999, MATSUMOTO *et al.*, 1998].

In agreement with our results, several authors reported that SCoT, CDDP and DAMD marker techniques were able to provide more dependable diversity information compared to RAPD or ISSR techniques [AMIRMORADI *et al.*, 2012; LI *et al.*, 2013; POCZAI *et al.*, 2013].

Based on the calculated PIC and polymorphism percentage, the used markers were highly efficient for assessing diversity among studied varieties.

A high level of polymorphism (125 polymorphic bands) was detected using five CDDP, six DAMD, and seven SCoT markers, with an average of 5.3, 5.9 and 6.6 bands per primer, respectively.

Although, the source of diversity was different however, the rate of diversity for the three marker techniques

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was approximately equal suggesting primers could properly and similarly bind to different regions of the genome.

Based on results, the average genetic distance obtained by SCoT, CDDP, and DAMD (0.44, 0.42 and 0.44, respectively) was similarly high showing a relatively high genetic dissimilarity among studied varieties.

Table 3.

Total number of amplified bands (TB), polymorphism bands (PB), percentage of polymorphism bands (PPB) and PIC values in *rose* varieties as revealed by DAMD, SCoT, and CDDP markers.

Marker type	Primer	TB	PB	PPB	PIC	MI
DAMD	UPR–2F	4	4	100	0.31	1.25
	UPR–4R	8	8	100	0.43	3.42
	UPR–6R	7	7	100	0.42	2.93
	UPR–13R	9	9	100	0.43	3.9
	UPR–17R	6	6	100	0.41	2.46
	UPR–25F	4	4	100	0.26	1.03
	UPR–30F	4	4	100	0.36	1.43
	UPR–38F	5	5	100	0.37	1.84
SCoT	SCOT–1	3	3	100	0.33	0.98
	SCOT–2	8	8	100	0.42	3.4
	SCOT–11	4	4	100	0.34	1.37
	SCOT–13	11	11	100	0.44	4.82
	SCOT–22	3	2	77	0.25	0.5
	SCOT–28	10	10	100	0.44	4.42
	SCOT–36	8	8	100	0.45	3.59
CDDP	KNOX–1	4	4	100	0.37	1.48
	KNOX–2	2	2	100	0.25	0.5
	KNOX–3	6	6	100	0.39	2.32
	WRKY–1R	7	7	100	0.4	2.82
	WRKY–2R	7	7	100	0.37	2.78
	WRKY–3R	6	6	100	0.41	2.46

Discordance between dendrograms obtained by SCoT and DAMD with CDDP could be explained by the different nature of each technique, region coverage of genome by each marker, level of polymorphism and the number of loci [SOUFRAMANIEN and GOPALAKRISHNA 2004; GORJI *et al.*, 2011].

Our results were in agreement with the previous reports about clustering varieties using different marker systems in potato [GORJI *et al.*, 2011], chickpea [HAJIBARAT *et al.*, 2015] and wheat [HAMIDI *et al.*, 2014].

The MI, general rate of efficiency in discovering polymorphism [KHODADADI *et al.*, 2011], was different in three marker systems (Table 3).

On the other hand, an important property of a suitable marker system is its capacity to distinguish among different accessions.

Our study revealed that the resolving power of SCoT and DAMD primers is higher than CDDP primers.

These results were in accordance with previous studies [KHODADADI *et al.*, 2011].

This study has implications not only just for revealing the genetic diversity within the genotypes used, but also for the management of genetic resources and their uses in applied breeding programs.

Information about current genetic diversity permits the classification of our available germplasm into various heterotic

groups, which is particularly important to hybrid/cross–breeding programs in rose.

Up to now the classical breeding programs for rose at National Institute of Ornamental Plants (NIOP) has maily relied on beed morphological traits.

The current study concluded the importance of molecular studies (easy, rapid and informative markers) in detecting genetic variation among varieties in selecting diverse parents to carry out a new crossing program successfully.

SCoT markers produced large numbers of amplification products per genotype.

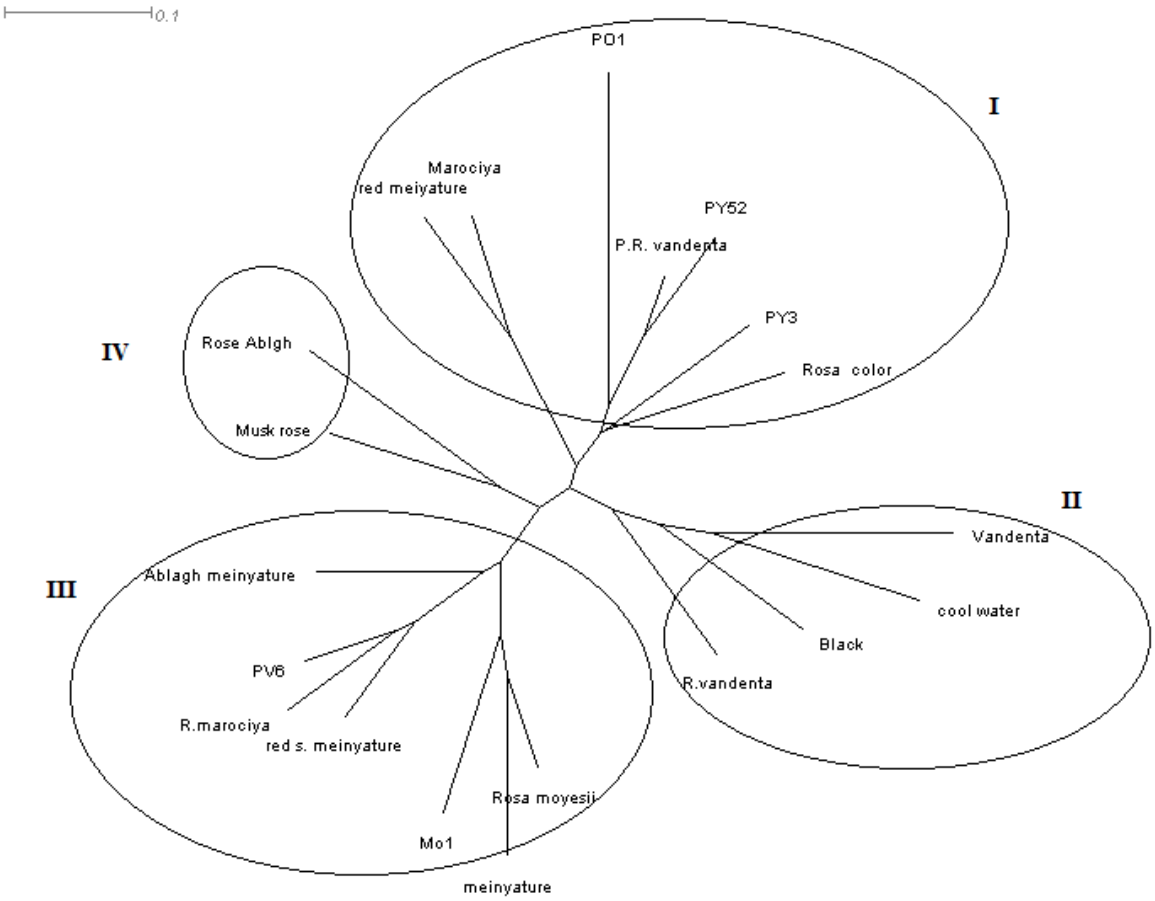

Figure 3. Dendrogram of the 20 local rose varieties using CDDP markers based on genetic distance.

Table 4.

Mantel test correlation coefficients among similarity matrices obtained using CDDP, SCoT and DAMD markers.

	CDDP	SCoT	DAMD
CDDP	1		
SCoT	0.92**	1	
DAMD	0.85**	0.89**	1

SCoT marker is a simple, low cost, and reproducible marker system compared with other marker systems, such as ISSR and SSR [GORJI *et al.*, 2011].

We propose that SCoT marker be used in combination with SSR or CDDP genetic analysis.

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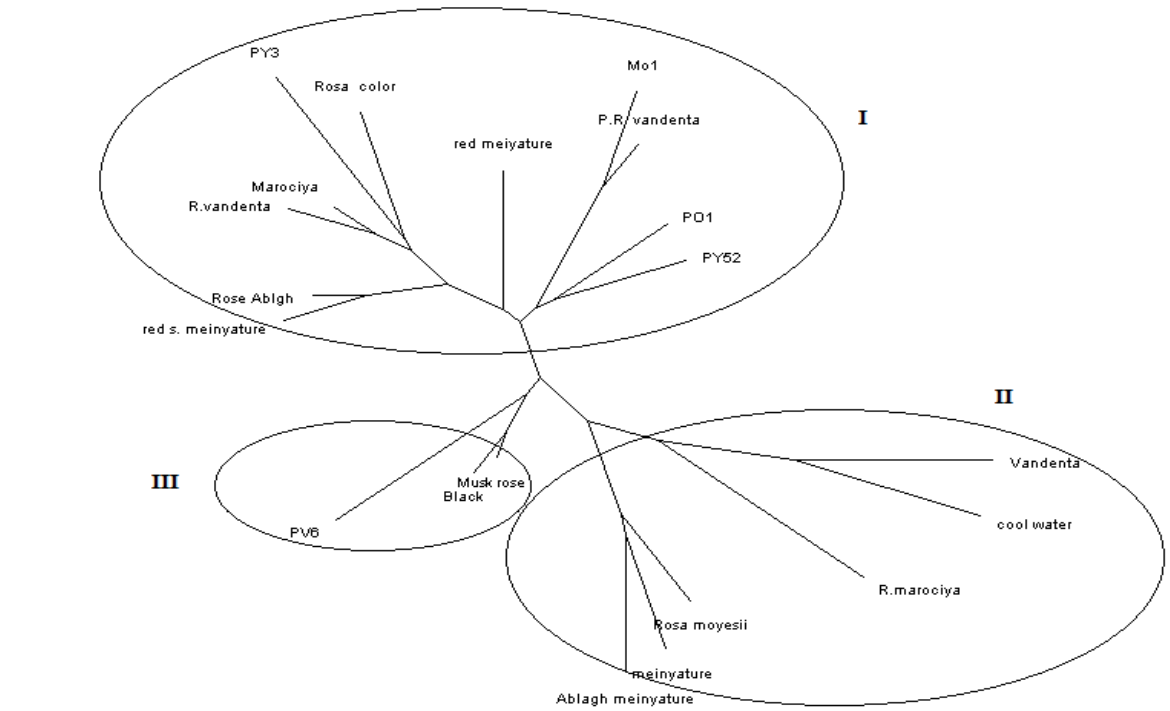

Figure 4. Dendrogram of the 20 local rose varieties using DAMD markers based on genetic distance.

Ornamental plants are heterogeneous and contain numerous groups of species.

Both genetic diversity and fingerprinting studies are of useful tools which enable plant breeders to make better decisions regarding selection of germplasm to be used in crossing plans [MILBOURNE *et al.*, 1997; RUSSEL *et al.*, 1997].

Conclusions

Our findings showed that SCoT marker analysis was successfully performed to evaluate the genetic relationships among the local rose varieties.

High polymorphism revealed by SCoT could be used for molecular genetics study of the rose varieties, providing high–valued information for the management of germplasm, improvement of the current breeding strategies, and conservation of the genetic resources of rose species.

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