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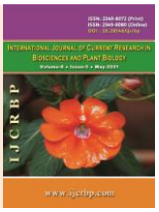

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Analysis of genetic diversity among different Rose (*Rosa*) genotypes using morphological and molecular markers (ISSR)

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Abstract

Rose is a woody perennial of the genus *Rosa* within the family *Rosaceae*. In this study, phylogenetic relationship among seven different genotypes of Rose (*Rosa*) was determined using ISSR primers and morphological parameters. DNA was isolated using CTAB method with 0.5% of CTAB concentration. Out of 50 ISSR primers, 16 primers gave reproducible banding pattern with all the seven genotypes of Rose while others failed to produce polymorphism. A total of 108 bands were obtained with an average polymorphism of 54.69% and an average of 9.06 bands per primer. The average similarity was found to be 0.69 among all the seven genotypes using Jaccard's similarity coefficient. On basis of polymorphism cluster analysis divided them into two major groups separating one genotype (light pink) from rest of the six genotypes and principle coordinate analysis confirmed the results. A parallel correlation was found between the morphological parameter (flower diameter) and the results of ISSR analysis as both of them divided the seven genotypes into two main groups.

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Introduction

Rose (the queen of flowers) is a woody perennial of the genus *Rosa*, within the family *Rosa*ceae grown all over the globe. Flowers of Rose vary in size and shape and are usually large and showy, in colors ranging from white through yellows and reds. As per reports in literature Rose is divided into four subgenera: *Hulthemia*, *Platyrhodon*, *Hesperhodos* and *EuRosa*, in which the first 3 subgenera include only few species. Mostly Rose species are highly variable and are hybridized easily, the classification of *Rosa* is sometimes difficult and the wild type of some modern Roses is not always known. The number of modern developed varieties has reached 25,000 with consistent increase in number which make it difficult to classify (Boronkay et al., 2000; Azeem et al., 2012). The genus *Rosa* comprises ~200 species, among which only 8–20 species have contributed to the genetic make-up of our present cultivars, namely the complex hybrid variety *Rosa×hybrida* (Reynders-Aloisi and Bollereau, 1996; Gudin, 2001). Each of these species may have contributed a specific trait. For example, *Rosa gallica* and other robust polyploid species lent the trait of cold hardiness, *Rosa chinensis* brought recurrent blooming, and *Rosa foetida* bestowed the yellow flower color. Uses of Rose are multifaceted and spread across different aspects like gardening, medicine, cosmetics,

cooking, essential oil production, commercial cut flower, for hedging and other utilitarian purposes (Akond et al., 2012).

Estimation of genetic diversity to identify groups with similar genotypes is important for conserving and utilizing genetic resources. Assessment of genetic diversity is done through various methods used either alone or in combination. These include morphological and molecular characterization, morphological traits are also essential for preliminary evaluation and for assessment of genetic diversity. Morphological characterization sets the basis for key traits of interest present in a germplasm collection as it is the simple way to assess variability in a collection (Khan et al., 2015; Jain et al., 2017).

Different types of DNA based molecular markers i.e. polymerase chain reaction (PCR) based markers, Non PCR or hybridization based markers and sequencing-based markers can be used to analyze the genetic diversity of plant materials. Molecular markers have been applied in different studies to evaluate genetic relationships of Roses (Niyazi et al., 2018). They represent a significant resource for creating genetic and physical genome maps for distinguishing individuals, investigating genetic relatedness and studying genome organization. Among various markers, Inter Simple Sequence Repeats (ISSR) are simple, quick and have become popular as their application does not need any prior knowledge about the target sequences in the genome (Godwin et al., 1997). Longer primer sequences and stringent PCR conditions makes these markers a choice for diversity analysis (Zhang and Dai 2010; Buldewo et al., 2012). Understanding of genetic diversity analysis and its distribution is essential for its conservation and use. It will help in determining what to conserve as well as where to conserve, and will improve our understanding of the taxonomy, origin and evolution of different plant species of interest.

Materials and methods

Morphological characterization and collection of leaf sample

Seven different Rose genotypes (Table 1) were taken in the present study for morphological and molecular marker analysis from different locations of Kurukshetra, Haryana and were used for DNA extraction as well as morphological characterization (Fig. 1). The assessment of morphological characters i.e. flower color, flower diameter, blooming period, stem height and flower shape were taken for measurement.

Fig. 1: Seven roses of different flower colors used for morphological and phylogenetic analysis based on ISSR.

Table 1. List of the seven Rose genotypes along with their important morphological characters used for analysis.

Flower color	Flower diameter (cm)	Blooming period	Flower shape	Stem height (cm)	Source / Location
Red	3.5	February-early May, Early September - early December	Star shaped	90-150	Rose garden Kurukshetra
Vibrant Yellow	3.5	February - early May, Early September - early December	Semi double cupped	60-90	Nursery Sec 7, Kurukshetra
Orange	2.5	February - early May, Early September - early December	Double bloom	70-110	Gurukul Kurukshetra
Yellow-White, Pink- White	2.5	February - early May, Early September - early December	Double cupped	50-90	Rose garden Kurukshetra
White	3.5	February - early May, Early September - early December	Single tosemi double	90-120	Rose garden Kurukshetra
Light pink	1.8	February - early May, Early September - early December	Double Tea shaped	80-110	Gurukul Kurukshetra
Dark pink	2.5	February - early May, Early September - early December	Star shaped	50-80	Gurukul Kurukshetra

DNA Extraction

DNA of all the seven genotypes of Rose was extracted from young leaf samples collected from different locations of Kurukshetra using CTAB extraction method of Murray and Thompson (1980), modified by Saghai-Maroof et al. (1984) and Xu et al. (1994). Leaves from seven morphologically different Rose (*Rosa*) genotypes were collected and powdered with liquid nitrogen. The powder obtained was thoroughly mixed with 10 ml of pre warmed 0.5% CTAB extraction buffer (Tris 1M, EDTA 0.5M, NaCl 5 M, β mercaptoethanol 0.2%, PVP 1%) and kept at 65 °C for 3 hours. Purified DNA was obtained by treating 100µl of DNA sample with 0.1 µl of RNase (10 mg/ml) and keeping at 37 °C for 10 min. Quality of DNA was determined by running DNA sample on 0.8% agarose gel along with the standard.

Analysis

The 0/1 matrix was used to calculate the similarity genetic distance using "SIMQUAL" sub-programme of NTSYS-pc software (Numerical Taxonomy and Multivariate Analysis System programme). Dendrogram was constructed by using distance matrix by the unweighted pair group method and arithematic average (UPGMA) a sub program of NTSYS-pc or gene profiler.

Polymorphic information content

The frequency of polymorphism between the genotypes was calculated based on the presence (taken as 1) or absence (taken as 0) of common bands. ISSR markers were used in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. PIC values are commonly used in genetics as a measure of polymorphism for a marker locus using linkage analysis.

Principle coordinate analysis

Three dimensional Principle component analysis was constructed for providing suitable means for testing the relationship among seven genotypes of Rose (*Rosa*) using the FAMD- Fingerprint Analysis with Missing Data Software.

Results

The present study was carried out to assess the phylogenetic relationship among the Rose genotypes by using morphological characters and inter simple sequence repeat (ISSR) markers. The assessment of morphological characters of all seven genotypes of Rose was done by visual observation. Flower diameter was assessed by using scale for all the seven genotypes of Rose. All the genotypes represented distinguishable

morphological characteristics (Fig. 1) and differed in flower color, flower diameter, stem height and flower shape. For flower diameter, three samples (Red, Yellow and White) belonged to large size flowers (3.5cm), three samples (Yellow- pink, Orange and Dark pink) belonged to medium size flowers (2.5cm) and the remaining one sample (light pink) belonged to small sized flowers (1.8 cm) (Table 1). All the seven genotypes formed three groups on the basis of morphological parameters (Flower diameter).

Out of 50 primers screened, 16 primers produced polymorphism where as others showed no amplification in any of the genotype. The highest number of polymorphic DNA fragments (8-10) were obtained by primer 1737-20 (Fig. 2) while, the lowest number of bands were generated with primer 9974-009. The percent polymorphism ranged from as low as 18.18% to as high as 88.88% (Table 2). The average polymorphism produced by sixteen primers was found to be 54.69. A total of 108 amplified bands were obtained using sixteen ISSR primers, out of which 52 bands were polymorphic and 56 bands were monomorphic. Total number of amplified bands varied from six to twelve and with an average of 9.06 bands per primer. A total of 13 alleles were generated and maximum number of alleles (three) was generated by two primers and minimum of one allele was generated by three primers (Fig. 3a). The allele size ranged from minimum 300bp in light pink to maximum 1220bp in yellow pink (Fig. 3b and Table 3).

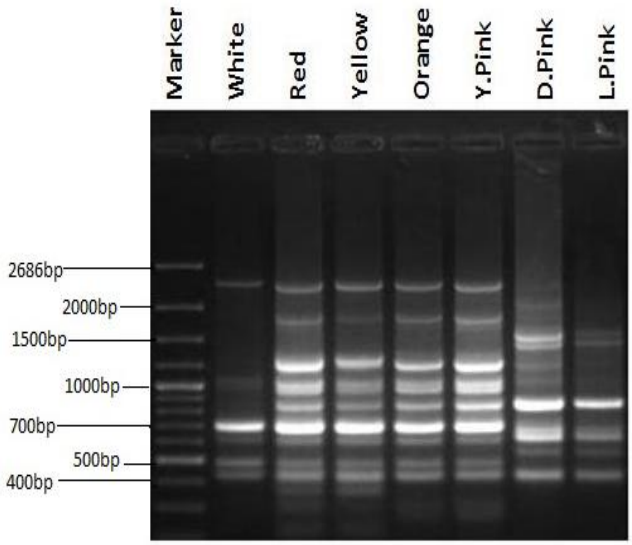

Fig. 2: Agarose gel electrophoretic separation of PCR amplified genotypes of Rose with primer 1737-020. Marker is Quantum PCR high range marker.

Table 2. DNA amplification bands and polymorphism generated in Rose (*Rosa*) genotypes using ISSR primer.

Primer	Genotypes amplified	Total bands	Polymorphic	Monomorphic	Percentage (%) Polymorphism
9974-051	$(CT)_8G$	7	4	3	57.14
9974-052	$(CA)_8G$	10	5	5	50
9974-053	$(TC)_8A$	10	5	5	50
9973-009	$(CT)_8RA$	6	2	4	33.34
9973-011	$(AC)_8YT$	8	2	6	25
9974-056	$(AG)_8T$	8	3	5	37.5
9974-062	$(CTTCA)_3$	8	5	3	62.5
9974-063	$(GGC)_6$	9	5	4	55.5
9973-008	$(GA)_8YC$	12	5	7	41.6
1737-026	$(GAA)_6$	10	6	4	60
1737-019	$(AT)_8T$	11	2	9	18.18
1737-024	$(GA)_8YC$	9	8	1	**88.88**
1737-025	$(GA)_8YG$	8	6	2	75
1737-020	$(TG)_8A$	12	10	2	**83.34**
1737-028	$(AC)_7$	7	4	3	57.1
1737-022	$(AG)_8YA$	10	8	2	**80**

Table 3. List of primers with unique alleles from different genotypes of Rose (*Rosa*) using ISSR primers.

Primer	No. of unique alleles	Allele size (bp)	Genotypes
9974-052	3	900, 850, 490	Yellow, White, Light Pink
9974-053	2	450, 300	Light Pink, Light Pink
9973-09	2	700, 600	Red , Light Pink
9974-062	1	300	Light Pink
9974-063	1	1000	Dark Pink
1737-026	3	1220, 1190, 400	Yellow Pink, Orange, Light Pink
1737-022	1	975	Red

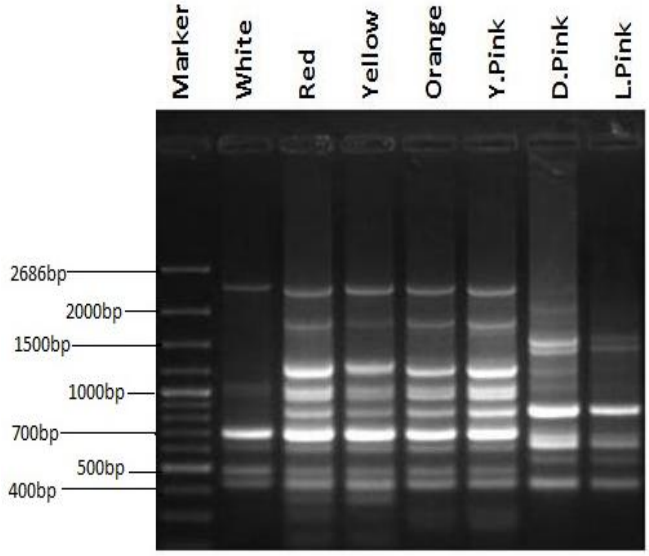

Fig. 2: Agarose gel electrophoretic separation of PCR amplified genotypes of Rose with primer 1737-020. Marker is Quantum PCR high range marker.

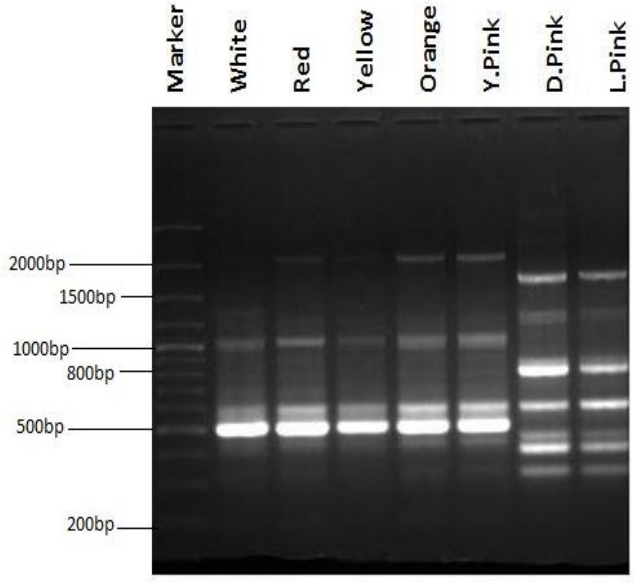

Fig. 3: (a) Agarose gel electrophoretic separation of PCR amplified genotypes of Rose with primer 1737-024. Marker is Quantum PCR high range marker.

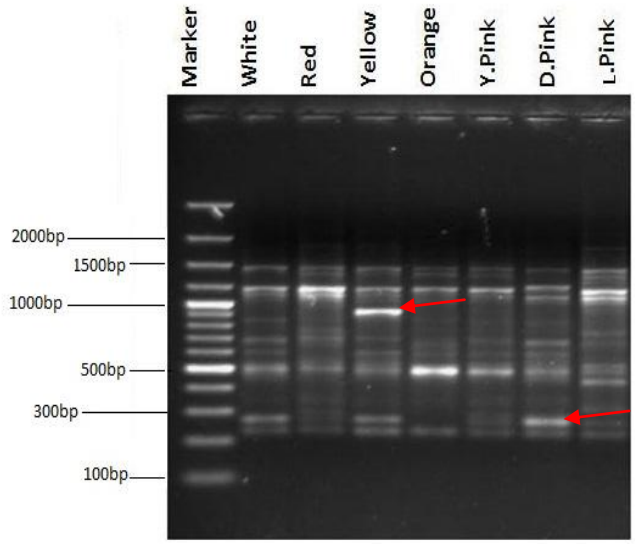

Fig. 3: (b) Agarose gel electrophoretic separation of PCR amplified genotypes of Rose with primer 9974-052 showing unique alleles (Arrows in red indicates unique alleles).

The 0/1 matrix was used to calculate the similarity genetic distance using "SIMQUAL" sub-programme of NTSYS-pc software. The matrix was constructed using Jaccard's similarity coefficient. The average similarity among all the genotypes was found to be 0.69 (Table 4). The similarity indices between different genotypes ranged from 0.50 (White- Light pink; Yellow- Light pink; Orange- Light Pink) to 0.89 (Yellow pink- Dark Pink; Orange- Yellow Pink).

Dendrogram was constructed by using distance matrix by the unweighted pair group method and arithematic average (UPGMA) a sub program of NTSYS-pc or gene profiler using Jaccard's similarity coefficient. The 0/1 binary data was used to construct the dendrogram. The dendrogram constructed depicts the relationship among 7 genotypes of Rose (*Rosa)* used in present investigation. The tree was divided into 2 clusters at similarity coefficient 0.53. Cluster I consisted of only light pink showing it to be genetically diversified from all other six genotypes.

Table 4. Similarity matrices of seven genotypes of Rose of ISSR computed with Jaccard coefficient.

	White	Red	Yellow	Orange	Y. Pink	D. Pink	L. Pink
White	1.00						
Red	0.85	1.00					
Yellow	0.80	0.87	1.00				
Orange	0.76	0.82	0.80	1.00			
Y. Pink	0.77	0.80	0.77	0.89	1.00		
D. Pink	0.74	0.80	0.75	0.83	0.89	1.00	
L. Pink	0.50	0.55	0.50	0.50	0.57	0.56	1.00

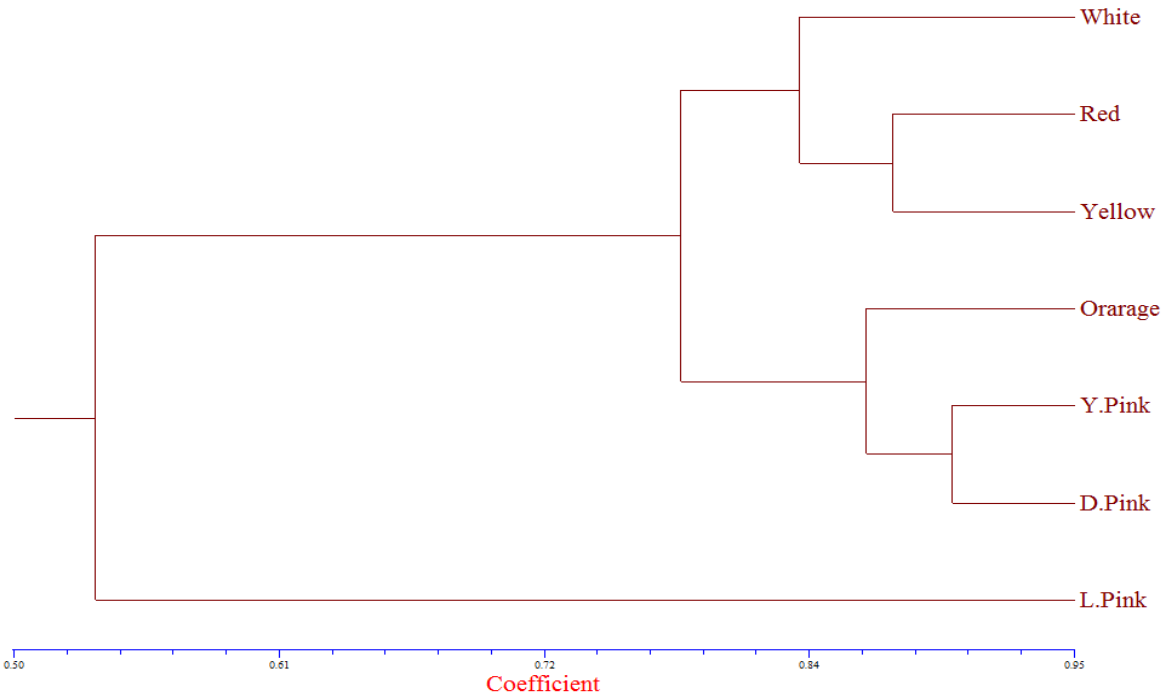

Fig. 4: Dendrogram (NTSYS-pc) constructed with UPGMA clustering method using ISSR primers for seven genotypes of Rose (*Rosa*) using Jaccrad's similarity coefficient.

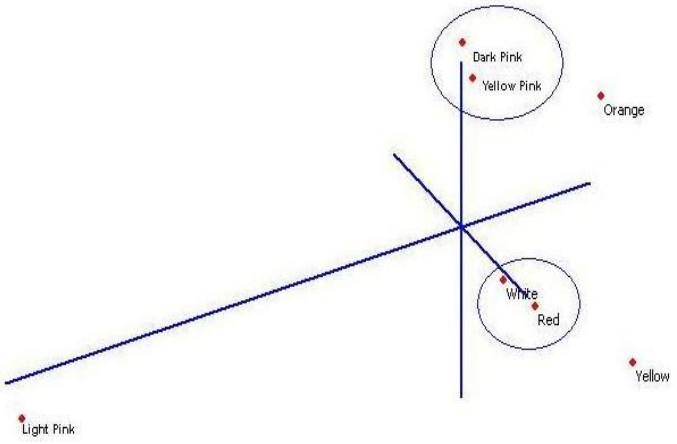

Fig. 5: Principle coordinate analysis of seven genotypes of Rose (*Rosa*) using ISSR primers.

Light pink was genetically unique and out grouped from rest six genotypes at a similarity coefficient of 0.53. Cluster II was occupied by all other six genotypes which got further divided into two sub clusters at similarity coefficient 0.78 into two sub clusters (Fig. 4). The sub cluster I got further divided into two sub sub clusters at similarity coefficient 0.87. Sub sub cluster I at similarity coefficient 0.90 was occupied by two genotypes Yellow-pink and Dark Pink. The sub sub cluster II was occupied by genotype Orange at similarity coefficient 0.87. The sub cluster II formed at similarity coefficient 0.78 was further divided into two sub sub cluster at similarity coefficient 0.82 and the sub sub cluster I got divided into two subgroups at similarity coefficient 0.88 which were occupied by two genotypes

Yellow and Red. The sub sub cluster II was occupied by White. The dendrogram based on similarity matrix revealed 50–89% genetic relatedness among seven genotypes of Rose (*Rosa*). The grouping/ clustering of 7 genotypes of Rose based on ISSR markers is in accordance to the cluster formed by the genotypes on the basis of their morphological characters (Flower diameter). Principle coordinate Analysis was done (PCoA) which supported results as shown by dendrogram and confirmed similarity between yellow pink-dark pink and white-red (Fig. 5).

Discussion

Morphological characters of almost all plants are affected by environmental conditions. Thus the use of morphological characters along with molecular studies can prove a better way for analysis. Productivity of a molecular marker technique depends on the amount of polymorphism it can detect among the genotypes under investigation. Inter-simple sequence repeats (ISSRs) have been widely employed to reveal levels and patterns of genetic variation in plants (Khalik et al., 2014; Sesli et al., 2020) to determine evolutionary relationships and to understand levels of genetic variation among wild and cultivated populations (Wu et al., 2006).

A parallel correlation was found between the morphological parameter (flower diameter) and the results of ISSR analysis as both of them divided the seven genotypes into two main groups in which one was individually occupied by light pink, separating it from the rest six genotypes which were grouped in the other cluster. There was no parallel correlation found between the morphological markers (petal color and blooming period) and molecular markers which may reflect the influence of environment on the performance of the materials. Wang et al. (2011) also suggested two reasons for low correlation between DNA markers and morphological markers first that most traits are governed and regulated by a number of genes where as each gene possibly governs and regulates more than one trait and secondly the binding site of each primer cover the whole genome and not only corresponding gene sections. Gitonga et al. (2014) also tried to classify garden rose in three groups-wild, old garden and modern garden rose on the basis of several morphological parameters.

Gitahi et al. (2016) used both morphological and molecular markers to assess the diversity of 36 local mango accessions from Kenya and reported high morphological diversity based on the fruit traits versus a low genetic diversity based on SSR markers. They reported that this difference is due to high environmental influence on the fruit traits and the proximity of the sampling locations to each other.

The genomic DNA in present study was extracted from freshly collected leaves of seven different Rose (*Rosa*) genotypes using CTAB extraction method given by Murray and Thompson, (1980) and modified by Saghai-Maroof et al. (1984). The composition of extraction buffer used for best quality DNA of Rose genotypes was: 0.5% (w/v) CTAB, 1M Tris, 0.5 M EDTA and 5M NaCl. Same molar concentration of Tris, EDTA and NaCl was used in the genomic DNA isolation for identification of genetic diversity among papaya varieties using morphological and molecular markers (Madarbokus and Ranghoo-Sanmukhiya, 2012; Singh et al., 2020).

In the isolation of Genomic DNA from *Hibiscus* species, mango Kadve et al. (2012) and Gebrehiwet et al. (2019) used 2% CTAB but here in case of Rose genotypes the CTAB concentration used was 0.5%, still obtained good quality of DNA which could be readily used for PCR. Lower concentrations did not show proper amplification and high concentrations showed decreased specificity and presence of smear that affected the repeatability (Sane et al., 2012). This was not exactly the case with Rose (*Rosa)* because low concentrations of DNA did not showed proper amplification but on increasing the concentration to 100 ng best and reproducible bands were obtained. Using 15 ng of DNA template reproducible banding patterns were obtained in the genetic diversity assessment on populations of three *Satureja* species in Iran using ISSR primers as reported by Kameli et al. (2013) which is very less than the one used in Rose (*Rosa*) but at this concentration (15ng) no amplification was obtained in case of Rose. Fifty ISSR primers generated a total of 108 reproducible and clear bands, out of which, 52 bands were polymorphic and 56 bands generated by the primers were monomorphic. Panwar et al. (2015) observed 94% of genetic polymorphism with ISSR markers then Prasad et al. (2006) who reported 87.5% of polymorphism in fragrant rose cultivars with RAPD markers. The present study is contrary to ISSR based polymorphism obtained in several other plant species *viz.*, 97.10% in *Atriplex halimus*, 96.68% in *Diospyros* (Deng et al., 2015), 95.71% in *Quercus brantii* (Alikhani et al., 2014).

The percent polymorphism gave the idea of polymorphic content in a given genotype. The percent polymorphism in Rose (*Rosa*) was found to be 54.69%. Kumar et al. (2011) found 86.1% of polymorphism in ISSR analysis of *Artemisia annua*. Seventeen primers produced 85 bands out of which 78 were polymorphic with an average of 4.58 polymorphic fragments per primer. The amplified bands in seven genotypes of Rose (*Rosa*) using 50 primers varied from 6 to 12 per primer with an average of 9.06 bands per primer which is nearly comparable with earlier findings by Panwar et al. (2015) who reported an average of 11 bands per primer in 32 rose cultivars with ISSR primers while polymorphism generated by RAPD marker is not comparable to our work as it gave only 6.5 bands per primer.

The PIC values are commonly used in genetics as a measure of polymorphism for a marker locus using linkage analysis and PIC is used to estimate the overall utility of each marker system. PIC provides an estimate of discriminating power of a marker. Kameli et al. (2013) used PIC values for analysis of diversity in *Satureja* genus which ranged from 0.22 to 0.36. Similarly in case of Rose (*Rosa*) PIC values ranged from 0.138 to 0.244 indicating low level of polymorphism among Rose (*Rosa*) genotypes.

The similarity indices for different genotypes ranged from 0.50 to 0.89. The average similarity across all the genotypes was found to be 0.69 indicating a moderate level of genetic similarity among the genotypes. The tree was divided into 2 clusters at similarity coefficient 0.53. Cluster I consisted of only light pink showing it to be genetically diversified from all other six genotypes. Light pink was genetically unique and out grouped from rest six genotypes at a similarity coefficient of 0.53.

The dendrogram constructed depicts the relationship among 7 genotypes of Rose (*Rosa)* used in present investigation. The tree was divided into 2 clusters at similarity coefficient 0.53. Cluster I consisted of only light pink showing it to be genetically diversified from all other six genotypes.. PIC values of 16 ISSR primers ranged from 0.138 to 0.244 with an average of 0.19 per primer. From the present study, we believe that ISSR and morphological approaches should be combined in order to estimate the extent of genetic diversity and generating valuable information for use in further conservation measures.

Conclusions

From our study, we conclude that data generated by using morphological and ISSR marker provided valuable information which can be used for further breeding program. However, in order to generate more specific and reliable information further studies can be carried by using co-dominant molecular markers.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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