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Hybrid identification, morphological evaluation and genetic diversity analysis of *Rosa* × *hybrida* by SSR markers

Muhammad Nadeem[1], Xinwang Wang[2], Masum Akond[2], Faisal Saeed Awan[3*], Azeem Iqbal Khan[3], Atif Riaz[4], Adnan Younis[4]

[1]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
[2]Texas AgriLife Research Centre, Dallas, Texas, USA. Department of Horticultural Sciences, Texas A&M
[3]University, College Station, Texas, USA
3Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan
[4]Department of Horticultural Sciences University of Agriculture Faisalabad, Pakistan

***Corresponding author: awanfaisal@yahoo.com**

Abstract

In rose breeding, identification of the parents based on the morphological characteristics becomes sometimes very difficult, and the molecular techniques open new horizons for research and improvement in this crop. The present study based on the F_1 cross of the 9 hip-bearing parents which produced 22 hybrids. Morphological evaluation of parent and hybrids included various qualitative traits like leaf color, hairiness, margins etc. Significant variation was revealed among parents and hybrids based on all parameters investigated including differences in overall performance. Genetic diversity among parents and verification of hybrids was evaluated using 10 polymorphic microsatellites markers. Estimates of heterozygosity varied among SSR loci and overall values of observed heterozygosity were 0.887 and total gene diversity was 0.852. Genetic relationship was established among all 32 genotypes by UPGMA revealed that narrow genetic base was found among the genotypes. All of hybrid and parents also showed allele homology with each other, reflecting the involvement of at least one parent genetic background in the cross. Inheritance of parental alleles was used to confirm the heterozygous nature of hybrid progenies.

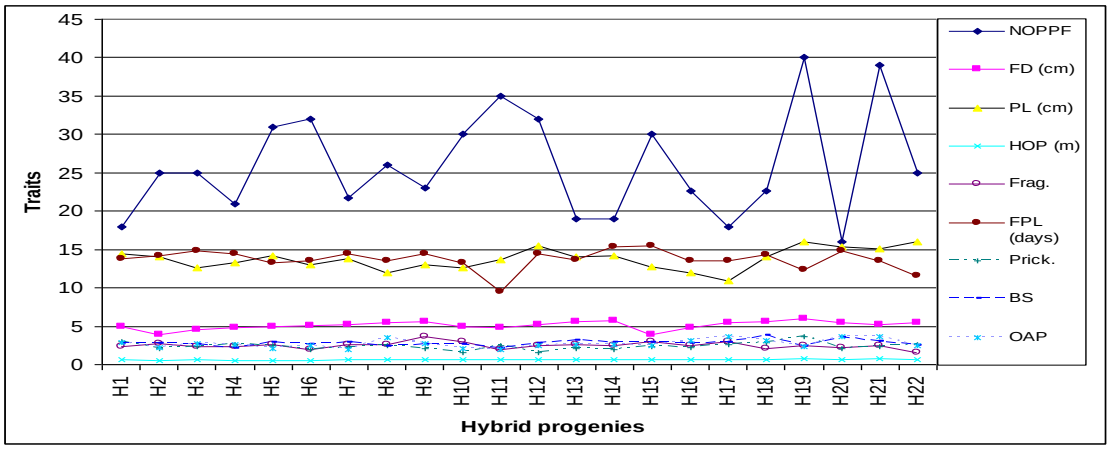
Keywords: Microsatellites; Rose Hybrids; Molecular Markers; Molecular Characterization.
Abbreviations: AFLP_Amplified fragment length polymorphism; CTAB_Cetyl trimethylammonium bromide; QTLs_Quantitative trait loci; RAPD_Random amplified polymorphic DNA; RFLP_Restriction fragment length polymorphism.

Introduction

Roses are an important economic crop worldwide as they are cultivated for cut flowers, essential oil and landscape use. Existing rose cultivars lose usefulness due to changes in environmental conditions, disease and insect pressure, and biotic stress. Hence, there is always need for genetically improved cultivars. Rose breeding provides a consistent source for development of new cultivars and unique germplasm for the garden and cut flower industry. In conventional breeding, progeny selection is made on the basis of superior morphological attributes. Breeding can be further aided by using molecular markers to see the genetic similarities and differences among species and cultivars because molecular markers provide a reliable tool to analyze the polymorphism among parents and progenies (Rusanov et al., 2005). Molecular markers are valuable tools for the screening and selection of desirable genotypes. Because of this considerable efforts were put on the use of these markers for variety identification in roses, such as RFLP (Ballard et al., 1995), AFLP (Basaki et al., 2009), RAPD (Riaz, 2006) and SSR (Wang et al., 2011). Molecular markers have also been used to monitor introgression and mapping of QTLs (Oyant et al., 2008), and marker-assisted selection (MAS) of cultivated roses (Debener et al., 2003). In certain circumstances, rose hybrids are difficult to distinguish based on morphological characteristics alone, presumably because of intra- and inter-specific crossing within the genus *Rosa* (Millan et al., 1996). Molecular markers have also been used to verify identification of breeding lines, hybrids and cultivars (Smulders et al., 2009). Many private companies are involved in rose breeding and have developed cultivars that are protected by plant patents. Among molecular markers, SSR are useful because they are codominant, can detect a large number of alleles, are able to discriminate between closely related individuals efficiently (Babaei et al., 2007) and verify hybrids (Iqbal et al., 2010). Because SSRs are reserved between closely related species, they could provide a marker database for cultivar identification and can be useful in rose genetic and population analyses (Zhang, 2003). SSR analysis can be performed by various techniques such as traditional agarose, polyacrylamide and capillary electrophoresis (Wang et al., 2009) but capillary electrophoresis has advantage over other techniques as it is easy and less time and labor consuming, and cost-efficient. Accurate separation and estimation of allele size can be automated (Shi et al., 2003; Huang et al., 2006: Wang et al., 2009). The present study is aimed to evaluate the progenies after crossing among *Rosa x hybrida* cultivars and to check the morphological differences for superiority over parents. Genetic diversity was analyzed by SSR, which was used to

Table 1. Parent cultivars and hybrid progenies (F_1) used in this study

Parent Cult.	Cultivar Name	Hybrid Progeny	Cross	Hybrid Progeny	Cross	Hybrid Progeny	Cross
V1	'Autumn Sunset'	H1	♀V1 × V3♂	H9	♀V7 ×V1♂	H16	♀V6 ×V8♂
V2	'Ice Berg'	H2	♀V1 × V4♂	H10	♀V9 ×V1♂	H17	♀V6 × V7♂
V3	'Paradise'	H3	♀V1 × V7♂	H11	♀V3 ×V4♂	H18	♀V7 × V9♂
V4	'Angel Face'	H4	♀V1 × V5♂	H12	♀V3 ×V7♂	H19	♀V7 × V8♂
V5	'Casino'	H5	♀V1× V6♂	H13	♀V3 ×V8♂	H20	♀V8 × V6♂
V6	'Louise Odier'	H6	♀V3 × V1♂	H14	♀V4 ×V8♂	H21	♀V8 × V9♂
V7	'Grand Margina'	H7	♀V8 × V1♂	H15	♀V4 ×V5♂	H22	♀V9 × V7♂
V8	'Handel'	H8	♀V6 × V1♂	-----	--------	-------	-------
V9	'Gruss-an-Tepletz'						



NOPPF= Number of petals per flower; **LOP**= Length of petal; **PL** = Pinnate length (cm); **Frag.** = Fragrance; **FPL**= Flower persistence life; **BS** = Bush shape; **FD**= Flower diameter (cm); **OAP**= Overall performance; **Prick.** = Prickles

Fig 1. Comparison of morphological traits (growth parameters) of 22 hybrids.

confirm the contribution of each parent in the development of F1 hybrid population of roses.

The objectives of this study are to (1) phenotype rose interspecific hybrids based on their horticultural traits; (2) genotype these hybrids based on SSR assay; (3) estimate genetic diversity of these hybrids differing their parents.

Results

Morphological studies

All the parent cultivars and hybrids showed variations in their morphological traits. Among the parent cultivars, foliage color varied from dull green and glossy for cultivars 'Autumn Sunset' and 'Handel' to pale green. The most attractive foliage was exhibited by cultivar 'Ice berg' with excellent light green, glossy foliage with sharp apices. Leaf margins of all cultivars varied from un-serrated to highly serrated. Leaves of the cultivars 'Casino' and 'Autumn Sunset' were slightly serrated as compared to cultivars 'Paradise', 'Angel Face', 'Louise Odier', 'Grand Margina' and 'Gruss-an-Teplitz', which have highly serrated leaves. Leaf hairiness and petiole pubescence was observed in the cultivars 'Autumn Sunset', 'Angel Face', 'Casino' and 'Gruss-an-Teplitz'. Leaf hairiness and petiole pubescence was absent in the cultivars 'Ice berg' and 'Louise Odier'. Observation of flower color resulted in a wide range of variation. Flower color of each cultivar was estimated by comparing with color chart (citation). Most of the cultivars exhibited clustered inflorescence showing bunches of flowers on a peduncle. The cultivars 'Autumn Sunset', 'Ice Berg', 'Angle Face', 'Casino', 'Louise Odier', 'Grand Margina', 'Handel' and 'Gruss-an-Teplitz' showed clustered inflorescence. In cultivar 'Paradise' solitary inflorescence. Among the hybrids, variations were also recorded in the field evaluations. Result regarding various morphological traits of F1 progenies is presented in Fig-1.

Molecular studies

Estimates of genetic diversity varied remarkably among loci. Observed heterozygosity (H_O) based on Nei's estimation revealed that the locus H10D03, CL2996 and Rw54N22 showed the most variation (H_O = 1.000) and the highest number of alleles (16 or 17, respectively) (Table 4). In contrast, locus H23O17 (H_O = 0.508) and H22F01 (H_O =

Table 2. List of the SSR Primers used in the study.

Sr. No.	Codes	Sequence	Sr. No.	Codes	Sequence
1	Rh48F	GATAGTTTCTCTGTACCCCACCTA	15	Rw52F	GGCAGTTGCTGTGCAGTG
	Rh48R	TTGACCAGCTGCAACAAAATTAGA		Rw52R	TTGTGCCGACTCAAAATCAA
2	Rh78F	AAAGAAACGCGAAATCTATGATGC	16	Rw54F	CTCAACTTCCCCGCCTTATC
	Rh78R	TCTGGATGGGATTTAAAAGACAGG		Rw54R	CTCGGCAGCTCCACTATCTC
3	Rh80F	CATGCCAAACGAAATGAGTTA	17	Rw55F	CGGTGGTTGGACATTAAAGC
	Rh80R	TTATCTAAAGGGCTGCTGTAAGTT		Rw55R	GGAGGCAACAGCACACTCTC
4	RhB510F	AAACGATAGGTGAATCTGTGGGT	18	Rw59F	GGAAGGTGCGTATGCAAAAT
	RhB510R	CACTCAACCTTGTCCACTCCTAAT		Rw59R	GAGAGGCTCATGCGCTTTAT
5	155F	GAAAAGAACGAGGGGTTTCC	19	C172F	ACAACACCAACTAGAACTTGAGC
	155R	ACGGTCGGTAATCAAGATGC		C172R	GCTCAACAGCAACAACCTCA
6	69E24F	TCAGGTGGGTGAGCTTCAAT	20	CL2F	GAAGCAGGGAAGATCCATGA
	69E24R	TGATTAGCTTGCCGGTTCTT		CL2R	GGCCCAATGCTCACACTAAT
7	RMS015F	TAATGTAGGCAGATATAAAGGAGT	21	CL9F	GCCACCATAGCCAGAGACAT
	RMS015R	GCAGCTGCACAACAAGGAA		CL9R	GGGCAGAGAAGAAGTTGACG
8	Rw5F	TGGTTTGGGGTTTTGTGTCT	22	CTGF	GTCATCAAGGAGGACCAGGA
	Rw5R	GCACAGTCTCCACCTGACAA		CTGR	GATCAGCGACCACCATGTC
9	Rw12F	CAGTGTCCATGCTGACGAGT	23	H2F	TGGCCAACCTCTCTCTGTCT
	Rw12R	TGCTCCTGTTTTCTCTTTGCT		H2R	TCCCAGCTTCGCTTTGTTAT
10	Rw15F	CGGCTAGCAATCAGTGACAA	24	H5F	CACAGAAACGAAGCGCAGTA
	Rw15R	GGTCTTCCCTAATGCCCAAT		H5R	GCTCGAAGAAGTCCTGGATG
11	Rw16F	CCAACAAACACGAGGAATGA	25	H10F	CAATTCAAAACCACCGCTCT
	Rw16R	CCACACTGATGTTCCAGCAC		H10R	CGCAGAGTCAACGAACCATA
12	Rw20F	TTCCTCTTCTCCTCCCTCGT	26	H20F	CCCCTCCCTCTCTCTCTACAA
	Rw20R	AGCAGTTTCCTGGCGAGTTA		H20R	TGATGAGGTTGTTTGCGATG
13	Rw23F	TGCATTCATCCCTCTCACTG	27	H22F	ACCATTTCCGAGCGACTCTA
	Rw23R	TCAAATGCATGCTGAAAGGA		H22R	GAGGAGGAGGTGTGAATGGA
14	Rw34F	CTCCTTTAGACTCGGGACCA	28	H23F	ACACCAAGCAAACCAAAACC
	Rw34R	CAGGCACGCCATTTCTAACT		H23R	AGCACGAAAACCGAGAGAGA

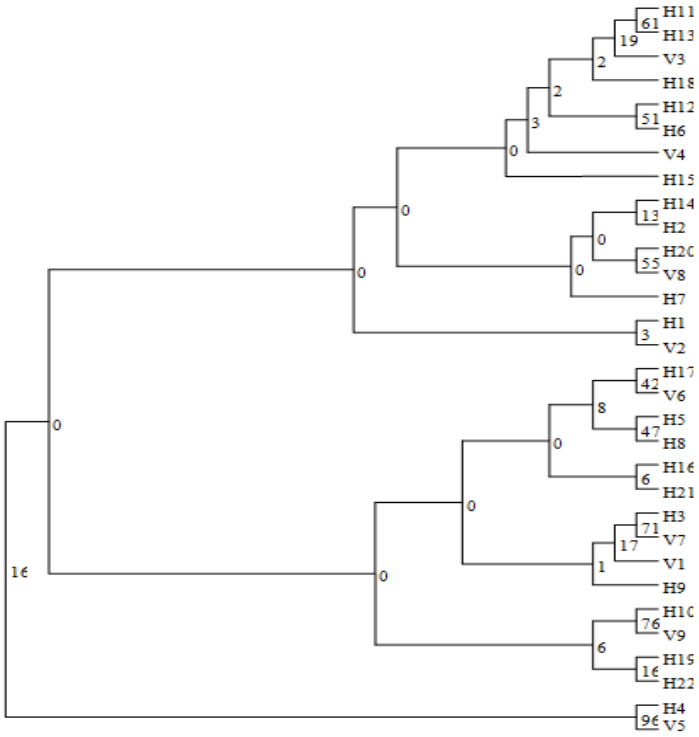


Fig 2. Dendogram showing relationship among parent cultivars and hybrid progenies.

Table 3. Characterization of morphological traits of parent cultivars.

Leaf traits/ Cultivar	'Autumn Sunset'	'Ice Berg'	'Paradise'	'Angel Face'	'Casino'	'Louise Odier'	'Grand Margina'	'Handel'	'Gruss-Antepletz'		
Leaf color	Dark green, glossy	Pale green, glossy	Dark green, semi glossy	Dull green	Pale Green, glossy	Light green, glossy	Dull Green	Dark green, glossy	Light green, pink blush, glossy		
Leaf Margins	Partially serrated	Un-serrated	Serrated	Serrated	Partially serrated	Serrated	Serrated	Serrated	Serrated		
Leaf hairiness	+	-	-	+	+	-	-	+	+		
Petiole pubescence	+	-	+	+	+	-	+	-	+		
Floral Traits											
Flower color	Yellow with pink blush	Snow white	Lavender	Plum mauve	Yellow	Hot pink	Yellow with pink bush	Hot pink	Deep pink		
Inflorescence type	Clustered	Clustered	Solitary	Clustered	Solitary clustered	Clustered	Clustered	Clustered	Clustered		
Leaf traits/ Cultivar	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
Leaf color	Light green, dull	Dark green glossy	Pale green, semi glossy	light green	Pinkish green	Dull green, glossy	Light green with pinkish blush	Dark green, glossy	Pale green, glossy	Dull green,semi glossy	Pale green, semi glossy
Leaf Margins	Serrated	Un-serrated	Serrated	Partially Serrated	Partially serrated	Un-Serrated	Serrated	Serrated	Serrated	serrated	Unserrated
Leaf hairiness	+	-	+	+	+	-	+	-	+	+	-
Petiole pubescence	+	-	-	+	+	-	-	+	+	+	+
Flower color	Light Yellow	Medium purple	Lavender	Orange Red	Crimson	Hot pink	Light Yellow	Dark Red	Magenta	White, creamy	snow
				mauve				pink	pink		
Inflorescence type	Clustered	Solitary clustered	Solitary	Solitary	Solitary	Clustered	Clustered	Clustered	Clustered	Solitary	Clustered
					clustered						
Leaf traits/ Cultivar	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22
Leaf color	Dark green, dull	Dull green	Light green, semi glossy	Light green	Dark green	Light reen, semi glossy	Pale green	Pale green, glossy	Dark green, glossy	Light green semi glossy	Dark green, semi glossy
Leaf Margins	Serrated	Serrated	Partially Serrated	Partially Serrated	Serrated	Un-Serrated	Serrated	Serrated	Serrated	Serrated	Unserrated
Leaf hairiness	+	-	-	+	+	-	+	-	+	+	-
Petiole pubescence	+	+	-	+	+	+	-	+	+	-	+
Flower color	Magenta	Light Pink	Light yellow	Dark Red	White	Pink color breakage	crimson	Dark Red	Light Pink	Peach puff	Misty rose
Inflorescence type	Solitary	Clustered	Clustered	Solitary	Solitary	Clustered	Clustered	Clustered	Clustered	Solitary	Clustered Solitary
					clustered						

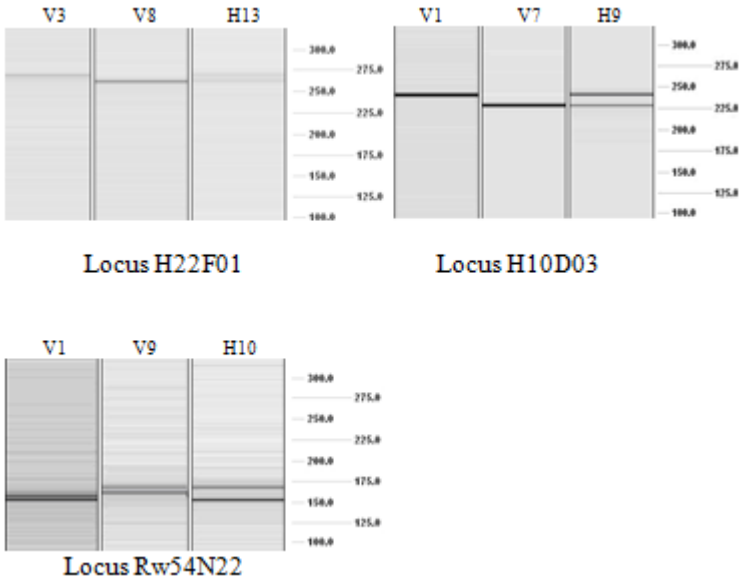


Fig 3. Amplification of common alleles among selected parents and hybrid (QIAxcel System, QIAGEN, USA).

0.652) exhibited to the least heterozygosity and fewer number of alleles (9). For all loci, observed heterozygosity (H_O) value was higher than that of expected heterozygosity (H_E). Overall comparison of clustering among parent cultivar and their hybrid samples is shown in Fig. 2a. The dendrogram from the genetic similarity matrix indicated that parental cultivars clustered into four groups but there is no significant bootstrap support. Confirmation of hybrids was done by comparing the common alleles between parent and hybrid against all primers (Fig-3).

Discussion

Evaluation of cultivars and progenies showed variation in their performance. These variations can be attributed to their genetic makeup (Bernier et al., 1993), environmental conditions like temperature, humidity and rainfall (Pettersen et al., 2006) and nutritional status of the bushes (van der Sart and de Visser, 2005). Environmental conditions may have produced misshapen flowers and less number of flower per bush because the temperature was over 32^{o}C, the humidity was down to 27% and rainfall was lower to 81mm in Faisalabad, Pakistan. Variation in morphological traits in roses was also studied previously (Kondo et al., 2005; Tabassum et al., 2002; Khattak, 1991; Farrante et al., 2010). The findings are similar to Hortus (1976) who stated that Hybrid Tea and Floribunda roses are the predominant garden and greenhouse cut-flower production roses with glossy foliage, recurrent blooming habit showy colors as described by Lammerts (1945). SSR loci have proven to be effective to identify parent cultivars and hybrids and to assess their polymorphic contents. However, the effects of missing data for H23O17 and RMS015 were not enough to reduce the resolving power of data so hybrid identification was effective. The existence of polymorphism showed by the selected primers can be attributed to recombination, mutations or random segregation of heterozygous chromosomes in the process of meiosis (Smith et al., 1996). Most of the cultivars used were tetraploid having same chromosome number hence allelic richness per locus showed little variation. The difference in allele richness can only partly be explained by the difference in ploidy level as *R. canina* is pentaploid and Hybrid Teas are tetraploid (Esselink et al., 2003). The high level of variation detected with the locus H10D03, CL2996 and Rw54N22 may be related to both outcrossing and the polyploidy nature of the parent rose varieties, which are proved by the high heterozygoty values evaluated by the majority of the selected markers. As expected, hybrids sharing alleles typically shared a common parent. For example, population group 1, 2 and 4 have cultivar 'Autumn Sunset' (V1) in their crossing. Some alleles were the same in both parents. One of the hypothesis supporting this lack of variation is that common parents may have contributed in the genome of the cultivars used as male and female parent in this study, hence it is proved that they share common alleles with narrow genetic background as compared to species roses and rootstock cultivars (Leemans and van der Laar, 1977). According to DeVries and Dubois (1996), Hybrid Tea varieties are the result of crosses within a limited gene pool and therefore a low degree of genetic variation may be expected. Based on the complementary banding patterns between the hybrid and their parents, the contribution of each male and female parent was confirmed as shown in Fig. 3. All the hybrid progeny showed sharing of common bands with their parents, although there were a few exceptions. This might be explained by the possible recombination and mutation in meiosis processing during hybridization (Huchett and Botha, 1995). Clustering among parent cultivars and F1 hybrids was based on Jaccard's similarity coefficient using UPGMA and showed that parent cultivars are similar. This grouping and similarities are due to continuous inbreeding in the development of modern cultivars. Although there are more than 200 rose species, only about seven to ten rose species are found in the background of most modern rose cultivars (Zlesak, 2006) so that they have narrow genetic background (DeVries and Dubois, 1996). In conclusion, SSR markers showed discriminative power to study variations among cultivars and their hybrids. Hybrids having one of the common parents also showed similarities with each other. Shared alleles within parents and hybrid confirmed the heterozygosity of hybrids for both male and female parents.

Table 4. Genetic diversity for all samples.

Locus	H_E	H_O	N
H10D03	0.837	1.000	17
Rw55E12	0.864	0.765	15
CL2996	0.797	1.000	17
C172	0.778	0.788	10
Rw54N22	0.812	1.000	16
RhB510	0.827	0.848	20
RMS015	0.664	0.712	15
H23O17	0.660	0.508	9
H22F01	0.672	0.652	9
Rw12J12	0.796	0.924	11
Mean	0.771	0.820	14

H_E= expected heterozygosity

H_O= Observed heterozygosity

D= Total gene diversity

Materials and Methods

Plant cultivars and hybrids

In current study, 31 genotypes (9 parents and 22 hybrids) were used, where nine hip-bearing hybrid rose cultivars were selected for the present breeding project as they previously performed better under climatic conditions of Faisalabad, Pakistan. They were hybridized according to a full diallel crossing scheme where all possible cross combinations were performed. Hips were harvested in August. The seeds were extracted and stored at 4 ^{o}C for 4 months. After cold dormancy, seeds were sown in peat moss in the greenhouse. Among all seedlings, early rouging was performed and 67 F1 progenies were selected and transplanted in the field after one year. 22 F1 hybrids (Table 1) were selected for comparison with parents as they were performing well in the field at the University of Agriculture, Faisalabad, Pakistan. Data for yield traits (Fig.1) was recorded on a weekly basis and pooled for monthly averages.

Genomic DNA isolation

Newly emerged leaves were collected from the selected progenies and parents during active period of growth for genomic DNA isolation. The leaves were preserved at -80˚C until DNA extraction and analysis. Leaf samples were lyophilized for transportation to Texas AgriLife Research and Extension Center, Dallas, TX, USA, where a CTAB method was used to extract genomic DNA (citation needed). In total, 22 hybrids were analyzed along with nine parent cultivars.

Microsatellite analysis

All the 31 genotypes were analyzed with 28 published SSR primers (Oyant et al., 2008; Yan et al., 2005; Whitaker et al., 2010). Of these 28 SSR primers, 10 primers showed polymorphisms and were selected for further analysis (Table-3). The PCR reaction volume was 10 µl consisting of 1 µl of genomic DNA, 2 µl of 5x GeneAmp PCR buffer, 1 µl of dNTPs (2 µM each), 1 µl of F/R primer mix (2 µM), 0.05 µl of AmpliTaq Gold® DNA polymerase (Promega, USA) and 4.95 µl sterile distilled water. To prevent evaporation during PCR reaction, one drop of mineral oil was added to the PCR mixture to cover the sample. The PCR thermal profile was 95^{o}C for 3 min, followed by 35 cycles of 94^{o}C for 40 s, 55^{o}C for 30 s, and 72^{o}C for 40 s, and a final extension at 72^{o}C for 1 min. PCR amplification was conducted in 0.2-ml 12-strip tubes using Mycycler Themal Cycler (BIO-RAD, USA). The PCR product was separated by capillary electrophoresis on QIAxcel System (QIAGEN, USA) using a 25-bp DNA step ladder to allow consistent allele size calling for all loci and samples tested.

Statistical analysis

Data for morphological traits was analyzed using statistical software SPSS 15 (SPSS, Inc., Chicago, IL). Means were compared by pair-wise Fisher's LSD (least significant difference) test at 1% level of probability. For the evaluation of fragrance (Frag.), prickles (Prick.), bush shape (BS) and overall performance (OAP), a rating scale was used with score 1 (having characters like few prickles, more fragrance, compact oval shape and overall good appearance) and 4 (having maximum prickles, low fragrance, irregular spreading shape and overall poor performance) (Fig. 1). For molecular studies, data from ten SSR loci were used for all samples. Hybrids were analyzed for shared allele frequencies with parent cultivars. Hybrids were verified on the basis of shared alleles for specific locus by comparing with their parents. Diversity within parent cultivars and hybrid progenies was calculated with respect to Nei's (1987) estimator of heterozygosity and unbiased gene diversity per locus using ATETRA Software (Van Puyvelde et al. 2010). The mean and standard deviation of different genetic variables can be calculated for expected heterozygosity (H_E) and obversed heterozygosity (H_O). Because rose cultivars and hybrids sampled in this study are tetraploid, up to 4 alleles were revealed in the SSR assay. We only scored two alleles to process cluster analysis of phenetic relationships using Populations 1.2.30 software (Langella, 2002). A dendrogram showing genetic similarity and differences among all groups was made by unweighted pair group method with arithmetic mean (UPGMA) with 100 bootstrap replicates (Saitou and Nei, 1987; Takezaki and Nei, 1996). Dendrograms were visualized with TreeView 1.6.6 (Page, 1996).

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