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Full Length Research Paper

Assessment of biodiversity based on morphological characteristics and RAPD markers among genotypes of wild rose species

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Conservation and utilization of the native plant resources is essential for long term sustainability of biodiversity. Wild native resources are adapted to specific and diverse environmental conditions and therefore, these adaptive features can be introduced into modern cultivars either through conventional breeding or advanced molecular genetic techniques. Understanding the genetic make up of the wildly growing plant species and of target desirable genes is a prerequisite for this purpose. Five wild rose (*Rosa* L.) genotypes were collected from different locations in northern hilly areas of Pakistan for this study. Different morphological characteristics and PCR based random amplified polymorphic DNA (RAPD) technique was used to find out the diversity and relationship among the genotypes. On morphological basis, *Rosa webbiana* collected from Muree and Nathia gali showed maximum (83%) similarity, whereas on DNA pattern basis, *Rosa brunonii* collected from Bansra gali and Sunny bank showed maximum (72%) similarity, while *R. webbiana* showed maximum diversity among all the species.

Key words: Genetic diversity, morphological differences, random amplified polymorphic DNA (RAPD), *Rosa*.

INTRODUCTION

Rose has been cultivated for the last 5000 years during ancient civilization of China, Western Asia and Northern Africa (Gudin, 2000), which facilitated its diversification. After selection and breeding for thousand years, especially after the first hybrid, tea roses were bred, rose became one of the most economically important ornamental crops. Many wild species of roses are endemic to Pakistan (Landrein et al., 2009), especially in the northern areas, which if improved through conventional breeding or advanced molecular techniques, can have great economic value for the people of the area, where farmers have small land holdings of less than 1 hectare and rely on conventional agriculture for making a living.

Biodiversity itself provides the basis for all life on earth, where land clearing and degradation are the one of the biggest threats to it. This vegetation clearing destroys fragments or modifies the habitats, and such activities contribute to further loss of biodiversity through accelerated land and water degradation (Anonymous, 2004). Loss of the specie or gene will result in reduced adaptive capacity (Savage, 2010). Conserving biodiversity, therefore, relies heavily on the protection of native vegetation in any area across the world, including areas strongly impacted by human activities (Hance, 2007).

Indo-Pak subcontinent has always been site of attraction for the whole world regarding its natural flora and diverse wild roses growing there are adaptable to several environmental stresses, which grow in cool temperate to hot arid regions. Native flora is expected to be adapted to diverse environmental stresses like disease, salinity, temperature, drought, nutrients, etc.

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and conservation of such plants require a broad understanding of biological diversity.

The genus *Rosa* L. belongs to the subfamily Rosoideae of family Rosaceae (Simpson and Ogorzaly, 2001) and comprises more than hundred botanical (wild) species (Crespel and Mouchotte, 2003). From many of the wild species, the large number of cultivated varieties and hybrids has been developed. Since many species are highly variable and hybridize easily, the classification of *Rosa* is sometimes difficult, and the wild type of some modern forms is not always known. On the other hand, incorporation of stress resistant genes into modern cultivars, both by using conventional breeding programs or modern molecular/genetic techniques, can be extremely useful in improving modern cultivars, because if species are more diverse genetically, there will be more possibilities of DNA encoding in it (Savage, 2010) and this will ultimately increase economic resources of the country.

To incorporate required attributes, it is essential to find out the genetic make up of these wildly growing plant species and their relationship with each other. Previously, some studies based on herbarium collections have been carried out for identification and classification of the wild roses growing in Pakistan in which morphological characters were considered for the identification and measuring of diversity (Maryum, 2000). However, genetic diversity of plants based on morphological traits is difficult to measure in natural populations because these traits are influenced by environmental factors to a large degree. To overcome this problem, PCR based molecular techniques have been used for genetic diversity estimations in many plants species (Debener et al., 2000a; Métais et al., 2000; Bredemeijer et al., 2002; Heckenberger et al., 2002; Allnutt et al., 2003; Awamleh et al., 2009; Mujaju, 2010; Panagal et al., 2010). Out of the various PCR-based multiple-loci marker techniques, RAPD, AFLP (Wim et al., 2008), microsatellite and SSR, are increasingly being used in this type of research. Among these, RAPD was largely used for fingerprinting and to estimate genetic relatedness in germplasm collections (Ebrahimi et al., 2009; Hasnaoui et al., 2010). In this particular study however, both morphological techniques and RAPDs were used to investigate genetic diversity in wildly growing roses collected from northern areas of Pakistan.

MATERIALS AND METHODS

Endemic wild roses were collected based on morphological differences from five different sites in the northern hilly areas of Pakistan including Muree foothills, Sunny bank, Ayyubia, Nathia gali and Bansra gali (Table 1). Sampling was conducted by transect method, where 10 permanent quadrants (5 m^2 each) were laid along the straight transect line, each separated by 20 m. The data were recorded during summer and winter seasons of the year. Morphological features of flowers, leaves, branches and fruits were recorded. Stem cuttings of wild genotypes were collected for future genetic studies in end June to early July, while fruits were collected in September. Cuttings of rose genotypes were wrapped in wet cloth, and brought down to Faisalabad and were grown in a mixture of soil and sand media in greenhouse of Rose Experimental Area, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, where temperature was maintained at 26°C.

Morphological studies

Plant samples of five genotypes collected for morphological studies were brought down to University of Agriculture, Faisalabad (U.A.F.) and identified by comparing with plants in the herbarium collection at Department of Botany, U.A.F. Data were recorded on the selected traits. Plant height was measured onsite from the base above soil surface to the tip of the branch and average of five longest branches was recorded, whereas five fully developed leaves from middle to bottom regions of plants were collected from the current year's growth. Total leaf length (cm) was measured from the apex to the base of the leaf along with leaflet length and leaflet number, while leaf colour was determined by comparing it with colour chart. Other leaf features including stipule shape, petiole pubescence, leaf hairiness, leaflet shape and leaflet margin were examined as per description given in Plant Form, An Illustrated Guide to Flowering Plant Morphology (Bell and Bryan, 1991). Twig hairiness and prickle shapes were also studied on branches. Flowers were collected from each plant in blooming period and flower colour, inflorescence type, calyx shape and corolla shape were recorded. Fruits (rose hips) were also collected and fruit shape and fruit length were measured, while fruit colour was examined by comparing it with colour chart.

DNA extraction for genetic studies

Three-week old leaves were collected from cuttings grown from all five genotypes and directly frozen in liquid nitrogen. DNA was extracted using the Qiagen DNeasy® Plant DNA extraction Kit (Qiagen Ltd., Crawley, U.K.) according to the protocol (James et al., 2000; Griffin et al., 2002). Extracted DNA was run on 1% agarose gel electrophoresis for 15 min to observe quality of DNA. DNA samples which gave smear results were rejected and re-extracted.

RAPD analysis

Polymerase chain reaction (PCR) conditions were optimized for rose DNA to obtain reproducible amplification with RAPD. PCR conditions were optimized with respect to rose DNA concentration, primers, number of thermal cycles, denaturing, annealing and extension temperatures, *Taq* DNA polymerase concentration and $MgCl_2$ concentration in PCR. The final volume of the PCR reaction mixture was 25 µl containing 15 ng/ul DNA, lU/ul *Taq* polymerase (FERMENTAS INC USA), 2.5 mM dNTPs (FERMENTAS INC USA), decamer primer (Genelink Inc. USA); 3 mM of $MgCl_2$, 10X buffer. The DNA amplification was carried out in a thermal cycler (Eppendorf AG No. 5333 00839, Germany) with 40 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min, followed by a final incubation at 72°C for 10 min. A total of 54 random 10 base pair RAPD primers were obtained from Genelink Inc. (USA), out of which 27 were selected which yielded consistent amplification.

The RAPD fragments were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide (l0 ng/l00 ml of agarose solution) in 1X TBE buffer, 5 µl samples were loaded in each well,

Table 1. Geographical distribution of collected rose genotypes.

S/N	Rose genotype	Geographical region	Elevation (m)	latitude	longitude
1	*R.webbiana*	Nathia gali	2,501	34° 06' 35" N	73° 28' 08" E
2	*R.webbiana*	Murree	2,133	33° 54' 00" N	73° 24' 00" E
3	*R.brunonii*	Sunny bank	2,210	33° 38' 56" N	73° 13' 72" E
4	*R.brunonii*	Ayyubia	2,718	34° 03' 08" N	73° 35' 92" E
5	*R.brunonii*	Bansra gali	2,228	33° 90' 41" N	73° 36' 74" E

Table 2. Correlations between sites of rose collections for soil organic matter (%).

Correlation	*R. brunonii* (Ayyubia)	*R. webbiana* (Murree)	*R. brunonii* (Bansra gali)	*R. brunonii* (Sunny bank)
R. webbiana (Murree)	0.97 (0.02)*			
R. brunonii (Bansra gali)	0.98 (0.02)*	1.00 (0.00)**		
R. brunonii (Sunny bank)	0.99 (0.01)	0.99 (0.01)**	0.99 (0.01)**	
R. webbiana (Nathia gali)	0.95 (0.04)*	1.00 (0.00)**	1.00 (0.00)**	0.97 (0.02)*

Figures in parentheses show P-value; * = P < 0.05; * = P< 0.01.

along with 5 µl of 1 KB DNA ladder mix (BDH Chemicals, U.K.) in each end and run for l.5 h at 150 V. Bands obtained on gel were measured by comparing PCR product with DNA ladder mix. These reactions were repeated for three times and only consistent and bright DNA bands were counted as present (1) or absent (0). The ambiguous and light DNA bands were rejected in this study.

Data analysis

Morphological data were analyzed by using multivariate technique "Cluster Analysis" with the help of statistical software Minitab (version 13.1) (State College PA, USA). Data was standardized by using the Z score. Similarities were measured by using Euclidean distance. The analysis was done at 50% similarity by using hierarchical clustering to obtain complete linkage clusting dendrogram (Affifi and Clark, 1996; Hair et al., 2005). Tuky's T method (Zar, 2003) was used for pairwise comparison among rose genotypes whereas, genetic similarities among all pairs of rose genotypes were calculated and analyzed using Popgen software (ver 1.44) (Cambridge, UK). This similarity matrix was analyzed and clustered with UPGMA (unweighted pair group methods using arithmetic averages) algorithm to determine the genetic relationships among rose genotypes.

Soil analysis

Composite soil samples were collected from the rhizosphere of the selected plants at each site, from where the experimental material was collected. Soil samples were collected at four points per selected site from top soil, 0 to 15 cm, 15 to 30 cm and 30 to 45cm depth, and composite sample were prepared for each depth.

RESULTS

Soil analysis distribution of rose genotypes

Analysis of soil samples collected from the five rose plant collection sites showed that, the soils are predominantly sandy loam in texture and well drained throughout the entire root zone, with pH ranging from 6.23 to 7.5. The relationship between soil characteristics and rose genotypes was studied by determining the correlation coefficient among the sites. The correlation between any two sites for pH, ranging from -0.92 to 0.85 was statistically non-significant, while there was highly significant correlation between Sunny bank and Nathia gali (sites growing different species) at ECe. It was observed that all sites had significant/highly significant correlation for organic matter, indicating that this character was not species specific (Table 2). Soil, silt, clay and $CaCO_3$ contents showed a non-significant correlation between the sites irrespective of *Rosa* species growing there but there was strong negative correlation (-0.95) between Bansra gali (*Rosa brunonii*) and Murree (*Rosa webbiana*) at soil silt percentage. Soil sand percentage also showed overall non-significant effect, although it was found to be the same on some sites and the only perfect positively significant correlation was observed between Nathia gali (*Rosa webbiana*) and Ayyubia (*Rosa brunonii*).

Morphological studies

Based on morphological characters, it was found that five plant genotypes collected from different locations belonged to two different species (*R. webbiana* and *R. brunonii*) and these species belonged to sections *Cinnamonae* and *Synstylae,* respectively. Diversity among genotypes, based on morphological features, using complete linkage method can be seen in the dendrogram (Figure 1) and Table 3 shows that at 50% similarity level,

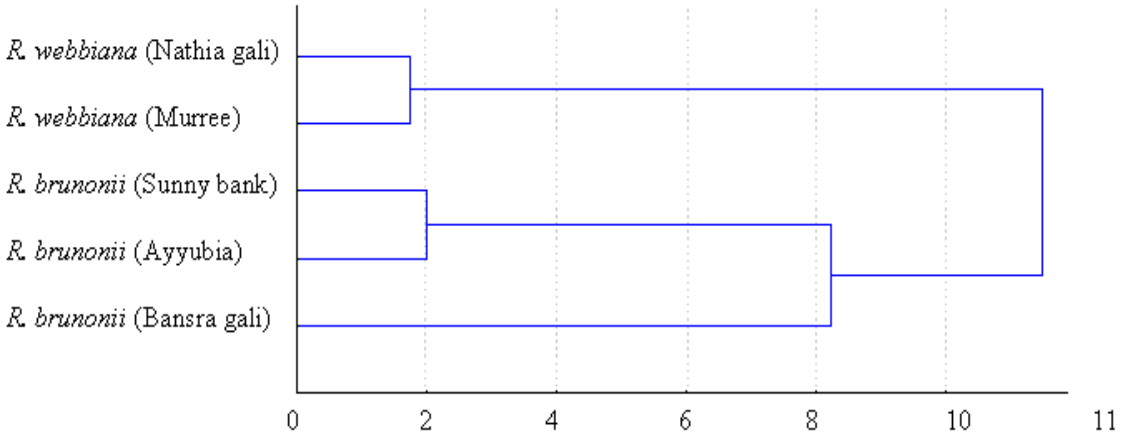

Figure 1. Complete linkage Euclidean distances dendrogram for similarities among rose genotypes based on morphological features.

Table 3. Euclidean distances for similarities among rose genotypes based on morphological features.

Rose genotype	*R. webbiana* (Nathia gali)	*R. webbiana* (Murree)	*R. brunonii* (Sunny bank)	*R. brunonii* (Ayyubia)	*R. brunonii* (Bansra gali)
R. webbiana (Nathia gali)	****	1.7	9.22	9.43	10.8
R. webbiana (Murree)		****	9.17	9.38	11.5
R. brunonii (Sunny bank)			****	2	8
R. brunonii (Ayyubia)				****	8.2
R. brunonii (Bansra gali)					****

there are two clusters. One of the clusters contains three genotypes of *R. brunonii* collected from Sunny bank, Ayyubia and Bansra gali, while the other cluster contains two genotypes of *R. webbiana* collected from Nathia gali and Muree. It is further noted that plants of *R. webbiana* collected from Nathia gali and Muree showed maximum similarity (83%) among all rose genotypes, while *R. brunonii* collected from Sunny bank and Ayyubia showed 80% similarity level.

RAPD analysis

List and sequences of RAPD primers are shown in Table 4. The genetic relationships among five rose genotypes based on RAPD can be seen in the dendrogram (Figure 2 and Table 5), using Nei and Li's (1979) similarity coefficient, where dendrogram clusters the genotypes mainly into two groups. To divide these genotypes into groups, 50% similarity (0.5 similarity coefficient) was taken as the cut off point. Dendrogram shows that *R. brunonii* collected from Bansra gali and Sunny bank showed maximum similarity (72%) among all collections followed by similarity index of 71% between the same species collected from Ayyubia and Bansra gali, and Ayyubia and Sunny bank (70%), respectively. Overall, *R. webbiana* particularly those collected from Murree, showed maximum diversity with all rose genotypes included in this study, which showed similar trend of least similarity (62%) with *R. brunonii* collected from Bansra gali and Sunny bank and even with *R. webbiana* itself collected from Nathia gali. *R. webbiana* collected from Nathia gali exhibited more similarity ranging from 63 to 65% with *R. brunonii* collected from various locations rather than the same species (*R. webbiana*).

DISCUSSION

Soil analysis distribution of rose genotypes

Soils collected from all selected sights are generally rich in organic matter which is much higher than the major soil series of Pakistan. These sites had non saline calcareous soils with high pH. However, there was always an acidic horizon in the root zone at all sites. Apparently, there was no consistent relationship between soil components and presence of these species growing in those sites.

Table 4. List and sequences of RAPD primers.

S/N	Primer name	Sequence	Amplified band/primer	Polymorphic band/primer	Percentage of polymorphic band (%)
1.	GLA-01	CAGGCCCTTC	6	3	50
2.	GLA-04	CAATCGCCGT	11	8	72.72
3.	GLA-12	GACCGCTTGT	9	7	77.78
4.	GLA-15	AGGTGACCGT	4	4	100
5.	GLA-16	AGCCAGCGAA	7	7	100
6.	GLA-18	AGGTGACCGT	12	9	75
7.	GLA-19	CAAACGTCGG	8	8	100
8.	GLA-20	GTTGCGATCC	11	9	81.81
9.	GLB-01	GTTTCGCTCC	9	9	100
10.	GLB-05	TGGGGGACTC	7	6	85.71
11.	GLB-11	GTAGACCCGT	10	8	80
12.	GLB-16	TTTGCCCGGA	9	9	100
13.	GLB-19	ACCCCCGAAG	9	8	88.89
14.	GLC-01	TTCGAGCCAG	8	7	87.5
15.	GLC-03	GTGAGGCGTC	10	9	90
16.	GLC-04	CCGCATCTAC	9	9	100
17.	GLC-05	GATGACCGCC	7	7	100
18.	GLC-06	TGTCTGGGTG	7	5	71.42
19.	GLC-07	AAAGCTGCGG	8	8	100
20.	GLC-08	GACGGATCAG	9	6	66.67
21.	GLC-11	AAAGCTGCGG	10	8	80
22.	GLD-10	GGTCTACACC	10	9	90
23.	GLD-13	TGAGCGGACA	6	6	100
24.	GLD-14	CTTCCCCAAG	12	9	75
25.	GLD-15	GGTCTACACC	2	2	100
26.	GLD-20	GGGACCTCTC	9	8	88.89
27.	GLF-17	AACCCGGGAA	10	9	90

Morphological studies

Morphological data have long served as major sources of information for inferring phylogenetic relationships among taxa and despite the emphasis on generating large molecular datasets that is currently seen in phylogenetics, morphological data remain both relevant and readily employed (Seth et al., 2010). Therefore, in this study, on the basis of 19 morphological characteristics, it can be suggested that genotypes of the species collected from different ecological environments did not exhibit much difference. However, the slight difference observed may be as a result of variations in environment that influenced characteristics like leaf length, plant height and fruit length. Some taxonomically important diagnostic features related to stem, leaves and inflorescence may be the consequence of adaptation to diverse environmental conditions, therefore, hunting native germplasm of *Rosa* and selection of promising genotypes can be immensely important for incorporating desirable characteristics in the future breeding efforts (Kazankaya et al., 2005). There were also considerable variations in leaves and shoots characteristics, fruit colour, hairiness, size and shape among the species and also within the accessions. Since most of these characteristics are used in the classification of *Rosa* genotypes, ecotypic variations in wild roses can effectively be identified and used in breeding research of modern cultivars (Kazankaya et al., 2005). Similar variations have been reported by several researchers in wild roses from different regions e.g., Kazankaya et al. (2005) in native genotypes of *R. canina* and Ercisli (2005) in *Rosa* spp. from Turkey, and Kiani et al. (2007) and Tabaei-Aghdaei et al. (2007) in *R. damascene* from Iran.

RAPD analysis

With the advent of polymerase chain reaction and modern molecular approaches to phylogenetics, DNA has become a major source for phylogenetic inference (Seth et al., 2010) and considering morphological data less important than DNA sequence data in phylogenetic studies is common (Endress 2002). Results based on

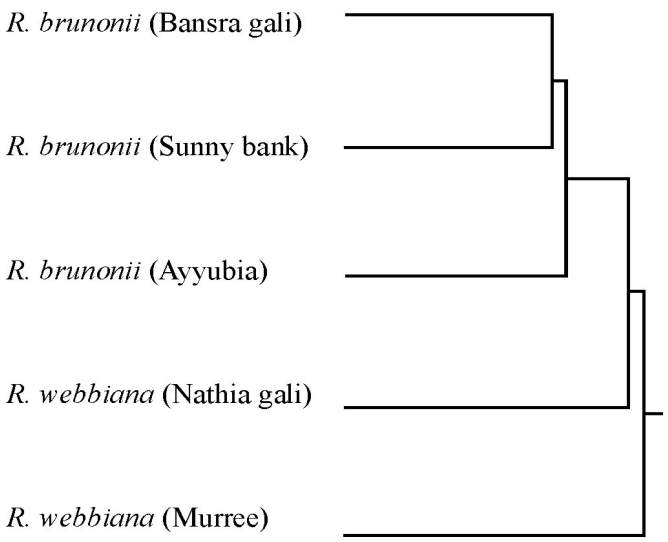

Figure 2. UPGMA dendrogram illustrating the genetic relationship among *Rosa* species based on Nei and Li's (1979) similarities at 197 RAPD bands.

Table 5. Nei and Li's similarity matrix index of five rose genotypes obtained from RAPD analysis.

Rose genotype	***R. webbiana*** **(Nathia gali)**	***R. webbiana*** **(Murree)**	***R. brunonii*** **(Sunny bank)**	***R. brunonii*** **(Ayyubia)**	***R. brunonii*** **(Bansra gali)**
R. webbiana (Nathia gali)	****	0.7232	0.7143	0.6339	0.6250
R. webbiana (Murree)		****	0.7054	0.6429	0.6250
R. brunonii (Sunny bank)			****	0.6518	0,6339
R. brunonii (Ayyubia)				****	0.6250
R. brunonii (Bansra gali)					****

morphological descriptions seem contradictory to the results based on RAPD, where genotypes of *R. webbiana* particularly collected from Nathia gali were found close to each other morphologically but genetically these are closer to *R. brunonii.* Morphological characters differ substantially from DNA sequence characters in their complexity and their frequency of evolutionary change (Harald et al., 2009). However, RAPD has been proved to be a useful genetic marker in taxonomic and genetic diversity studies (Kiani et al., 2007). These kinds of molecular/genetic markers can also be used to verify the origin of vegetatively propagated rose plants of doubtful origin (Debener et al., 2000b; Byrne et al., 2007). Data obtained from this study will be an excellent source for the genome mapping studies. The unique bands in each of these species will also be used for SCAR markers development (Sadia et al., 2007) (unpublished data).

Conclusions

It can be concluded that along with environmentally influenced characters, there were certain differences among genotypes which are related to the changes in genetic makeup of individuals, though similarity on the basis of morphological characteristics was more as compared to DNA based information. This diversity may be because of chance of hybridization among various wild species, which gives the opportunity to use these species together for further breeding program.

This can also be a very useful tool in rose crop improvement, which can help to generate rose varieties, more resistant to biotic and abiotic stresses. Apparently, there was no relationship between the soils characteristics and presence of a particular *Rosa* species in a site.

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