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Genetic diversity analysis in tuberose (*Pollianthes tuberosa*) genotypes through Randomly Amplified Polymorphic DNA

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Abstract

The present investigation was undertaken for characterizing tuberose genotypes using DNA marker technology. Twenty tuberose genotypes comprising of both single- and double-petal types collected from different parts of India were selected for analysis. The DNA extraction and RAPD conditions were standardized. For RAPD analysis 20 ng DNA template, 2.5 mM $MgCl_2$ and 1U *Taq*DNA polymerase was found effective. Out of 80 random decamer primers tested, 17 were selected based on high level of polymorphism. On the basis of primer resolving power and marker index, RAPD primers OPC-13 and OPD-12 were identified as efficient primers for diversity analysis of tuberose. A total of 157 RAPD bands were generated by the 17 random decamer primers. The selected primers proved effective for DNA profiling in addition to diversity analysis. Genotypes Guwahati Double and Swarnrekha showed good morphological similarity revealing high similarity coefficient (0.90) suggesting them to be very closely related. The RAPD analysis also confirmed their relatedness as they grouped in the same cluster. The suitability of this technique for genotyping and diversity analysis was also established. Genotypes, Vaibhav and Pune Single were found to have least pair-wise similarity although they had a greater morphological similarity with each other.

Key words: Genetic diversity, *Poliathes tuberosa*, RAPD, tuberose, germplasm

Introduction

Among the ornamental bulbous cut flowers, which are valued for their beauty and fragrance, tuberose (*Polianthes tuberosa* Linn. Family Agavaceae) a native of Mexico occupies a very special position. The long flower spikes are excellent as ideal cut flower. The loose flowers emit a delightful fragrance and are the source of tuberose oil. The genus *Tuberosa* contains 12 species of which nine have white flower [1]. In India, commercial tuberose cultivation is confined to one species *P. tuberosa,* which is basically a white-flowered type. Tuberose oil is one of the most sought after and expensive perfumery raw materials. The yield of concrete from fresh flower ranging from 0.08 to 0.11 per cent of which 18 to 23 per cent constitutes absolute.

Tuberose is diploid with chromosome number of 30, of which 5 are large and rest are small [2]. To meet the increasing demands for modern cultivar in the world trade, a large number of cultivars are being bred for novel and desired traits. Hence, accurate identification and characterization of different genotypes are essential for enforcing the intellectual property rights (IPR) of breeders. Characterization of accessions provides description of the material essential for their identification, conservation, management and utilization in crop improvement programmes. Molecular approaches collectively represent a potential tool that can be applied for effective characterization of germplasm. It addresses the limitations associated with morphological and biochemical processes [3]. Among the available molecular marker techniques, RAPD and ISSR are widely exploited by horticulturists, because results are obtained quickly and are fairly inexpensive to generate [4]. RAPD technique is simple, reliable, efficient and an economical means of cultivar identification and diversity analysis [5]. RAPD has been

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used successfully for genotype identification in several ornamental plants including *Lilium* species [6], alstromeria [7], heliconia [8] and gladiolus [9, 10].

Till date, very little attempts have been made on molecular characterization of bulbous ornamental crops. Molecular characterization of tuberose cultivars through DNA-based analysis is much desired, as there is much confusion in the naming of genetic material existing in various Indian states as they are exclusively referred to as single and double cultivars. It is probably expected that all the local single type genotypes have evolved from single genotype and all the local doubles probably from another. At present to our knowledge there is no report on the diversity analysis in tuberose using DNA markers.

Materials and methods

The experiments were carried out during 2007-08 utilizing 20 diverse tuberose genotypes collected from different states in India. A brief description of the genotypes maintained at Floriculture Farm, Division of Floriculture and Landscaping, IARI, New Delhi is given in Table 1. Young, fresh and healthy leaves were used for DNA extraction. The extraction of total genomic DNA was carried out as per method suggested by Sanghai-Maroof *et al.* [11].

DNA quantification was done using fluorimeter (Dynaquant TM 200, Hoefer Instruments, USA). The PCR reaction mixture consisted of *Taq*DNA polymerase**,** 10X PCR buffer, dNTPs, $MgCl_2$ (Bangalore Genei, India), 10-mer oligo-nucleotide primer (Operon Technologies, USA) and genomic DNA. Optimization of PCR component concentration was carried out for *Taq*DNA polymerase, $MgCl_2$ and genomic DNA. Concentration of primers (2 µm), dNTPs (2 mM) and PCR buffer (10X) was not varied. Optimization was done for the following components and factors. Mg^{2+} ion concentration was taken at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mM; template DNA concentration varied at 0, 5, 10, 15, 20, 25, 30, 35 and 40 ng; *Taq*DNA polymerase at 0, 0.25, 0.033, 0.5, 0.75, 1.0, 1.25 and 1.50 enzyme units. The annealing temperatures tested were 30, 32 and 35^0C. The PCR reactions was carried out in a thermocycler (Perkin Elmer, Massachusetts, USA) with following conditions, i.e. denaturation at 97^0C for 2 min. followed by 40 cycles of denaturation at 94^0C for 1 min., primer annealing at 35^0C for 1 min. and primer extension at 72^0C for 2 min. A final extension step was carried at 72 ^{0}C for 4 minutes.

Eighty decamer primers of A, B, C, D, M, O and P (Operon Technologies, USA) series were used to amplify genomic DNA of the randomly chosen genotypes, included in the study. Out of these, 17 primers that gave satisfactory amplification were chosen for further study (Table 2). The sequences of the selected primers are presented in Table 2. PCR amplification was carried out with 20 ng/µl of genomic DNA, 0.5 mM $MgCl_2$, 0.33 U/µl *Taq*DNA polymerase, 2.5X PCR buffer with 2 µM decamer primers and 200 µM of mix dNTP. A 1.8% agarose gel in 1XTAE buffer was prepared and the contents of the PCR tube were loaded into the gel. Electrophoresis was carried out at 60 V for 4 hours, i.e. until the bromo-phenol blue dye has traveled about 2/3rd the length of gel. The gel was then stained with 0.5 to 1.0 µg/ml ethidium bromide in distilled water for 30-45 minutes. The resolved amplification products were visualized by illumination under UV light from an UV-transilluminator (Vilbert Lourmat, France). The illuminated gel was photographed using gel documentation system (Bio-rad, Hercules, California, USA).

The amplification products were scored across the lanes comparing their respective molecular weights. Each band was treated as one RAPD marker. Presence of a band was scored as “1”, absence of a band as “0” and missing data was denoted by “9” using Jaccard’s similarity coefficient and was used to calculate similarity between accession pairs. The similarity matrix was subjected to cluster analysis by unweighed pair group method for arithmetic average (UPGMA) and a dendrogram was generated. These computations were performed using the program NTSYS-PC Ver. 1.8 [12].

Primer Resolving Power [12] was used to identify the primers that would distinguish cultivars most efficiently. Resolving Power (Rp) of a primer was calculated as the sum of “band informativeness” of all bands produced by a primer. Band informativeness (Ib) = 1 – [2(0.5 – p)],

Where, ‘P’ is the proportion of accession containing the band.

Resolving Power of the primer (Rp) is represented as Rp = ΣIb.

The information content of each primer was also determined. Marker index is the product of expected heterozygosity /gene diversity (Diversity index) and effective multiplex ratio (EMR).

$$\text{Diversity index} = \frac{\Sigma(1-\Sigma pi^2)}{n}$$

Table 1. Characteristics of tuberose genotypes selected for study.

Sl. No.	Genotype	Characteristics
1	Prajwal	Single flowers on tall stiff spikes, cross of Shringar x Mexican Single
2	Shringar	Single flowers on a sturdy spike a cross between Single x Double
3	Vaibhav	Semi-double flowers on medium spike, cross Mexican Single x IIHR-2
4	Calcutta Single	Flowers with one row of corolla segment
5	Calcutta Double	Flower with more than three rows of corolla segment
6	Hyderabad Single	Single flower
7	Hyderabad Double	More than three rows of corolla segment.
8	Pune Single	Flowers having single row of corolla segment.
9	Pune Double	Pure white flower with more than three rows of corolla segment
10	Pearl Double	Flowers are pure white with more than three segments of corolla
11	Subhasini	A multi-whorled variety developed from cross between Single x Double
12	Guwahati Single	Flowers having single row of corolla
13	Guwahati Double	Flowers with more than three rows of corolla segment
14	Saurashtra Single	Flowers with single segment of corolla
15	Saurashtra Double	Flowers with more than three rows of corolla segment
16	Phule Rajni	Single rows of corolla segment
17	Rajat Rekha	Single flowered type with silvery white streak in middle of the leaf blade
18	Swarn Rekha	Doubled flowered type with golden yellow streak along the margin of leaf blade.
19	Mexican Single	Florets bearing single segment of corolla
20	Sikkim Selection	Flowers are single but leaves are of variegated type

Where, 'pi' is the frequency of i^{th} allele and 'n' is the number of cultivars. Effective Multiplex Ratio = Fraction of polymorphic loci × No. of polymorphic loci for an individual assay.

$$\text{Fraction of polymorphic loci} = \frac{\text{No. of polymorphic loci for an individual assay}}{\text{Total number of loci for an assay}}$$

The Jaccard's similarity matrix was subjected to principal coordinate analysis. The coordination method makes use of multi-dimensional solution of the observed relationships. PCO resolves complex relationships into interactions of fewer and simpler factors. In this technique, the data matrix is derived from the distances (or similarities) between the operational taxonomic units. To simplify the description of these 'clouds' of points, the (principal) axes through the hyper-ellipsoid are calculated. The successive principal axes, representing the first major axis, the second axis etc., account for the greatest, the second greatest, etc. amount of variation.

Results and discussion

For effective resolution of the banding patterns, PCR reaction conditions need to be well defined to obtain reproducible banding patterns. The influence of various combinations of genomic DNA, $MgCl_2$ and *Taq*DNA polymerase on amplified products was worked out. On analyzing the amplified products on agarose gel, it appeared that concentrations of these components were the key factors that influence the banding pattern of the amplified products. At low concentration of template DNA, few bands were observed, whereas, at high concentration, smeared DNA patterns were observed. Out of the five different DNA concentrations; the best result was obtained with 20 ng. The optimum concentration of PCR components for tuberose RAPD analysis was 20 ng of template DNA, 1X PCR assay buffer, 2.5 mM of $MgCl_2$, 200 µM of dNTPs (dATP, dGDP, dCTP and dTTP), 1U of *Taq*DNA polymerase and 2 µM of primer. Similar findings were observed while working on poinsettia cultivars [13].

The concentration of $MgCl_2$ appeared to be important for obtaining clear bands as no bands was amplified in its absence. The relative intensities of amplified DNA fragments increased as concentration of $MgCl_2$ increased from 0.5 mM to an optimum of 2.5 mM beyond which, the band intensity decreased. The Mg^{2+} is known to affect the primer template interaction [14], polymerase activity and the melting temperature

Table 2. List of random decamer primers used for RAPD analysis

Primer number	Base sequence 5'-3'
OPA 9	GGGTAACGCC
OPA 13	CAGCACCCAC
OPB 13	TTCCCCCGCT
OPC 4	CCGCATCTAC
OPC 8	TGGACCGGTG
OPC 10	TGTCTGGGTG
OPC12	TGTCATCCCC
OPC13	AAGCCTCGTC
OPD 3	GTCGCCGTCA
OPD 5	TGAGCGGACA
OPD 8	GTGTGCCCCA
OPD 12	CACCGTATCC
OPD 13	GGGGTGACGA
OPM 5	CTCCATGGGG
OPM 10	TCTGGCGCAC
OPM 11	GTCCACTGTG
OPM 13	CCACACTACC

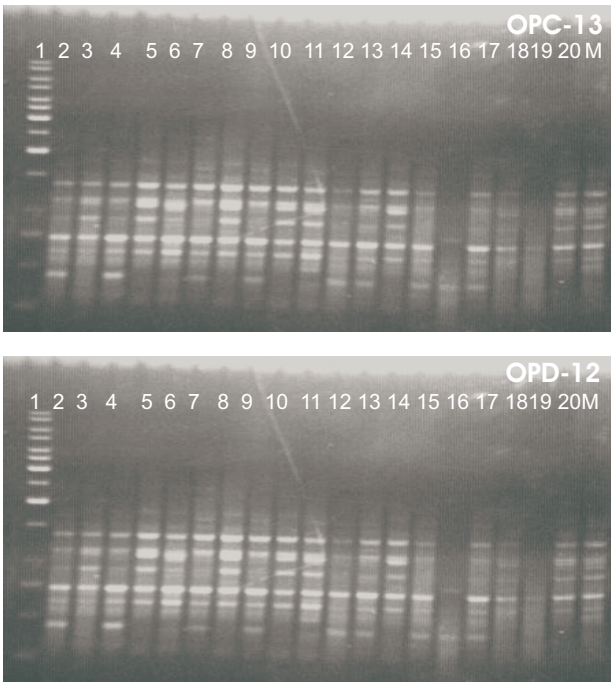

Fig. 1. Amplification profiles of tuberose genotypes employing random primer OPC-13, OPD-12 and OPM-11. Lane M: Marker, Lanes 1-20: Twenty genotypes

of double standard DNA. The optimum concentration of *Taq*DNA polymerase was found as 1U per 25 µl reaction volumes. Earlier it had been reported that variation of *Taq* DNA polymerase from 0.19 to 3.04 units influence the result only by limiting the polymerase level [15].

Out of eighty decamer primers 17 generated 157 bands and the size of the amplification ranged between 200 to 5,500 bp. The number of bands generated by each primer ranged from 6 (OPB 13) to 18 (OPC 13) with mean of 13 bands per primer. Out of the total 157 bands, 78 (48.40%) were polymorphic. The primer showing maximum number of polymorphic bands was OPC-13 (10 bands) followed by OPC-4 (7 bands). The polymorphism shown by different primers ranged from 10 to 66%. Primer resolving power and effective multiplex ratio were found to be highest for the primer OPC-13, while lowest was for the primer OPC 13 (Table 3). Maximum marker index (MI) was observed 1.00 for the primer OPC13 and diversity index (DI) was comparatively higher (0.18). Primer OPD 12 was found to have the highest diversity (0.20) index with comparatively higher value of marker index (2.42). From the above comparison, the primers OPC 13 and OPD 12 were identified as efficient primers for analysis of genetic diversity in tuberose. The primer with maximum polymorphic loci had comparatively higher amount of effective multiplex ratio and marker index with high resolving power.

Genetic relationships between tuberose accessions were determined on the basis of Jaccard's Pairwise Similarity Coefficients. Genotypes, Guwahati Double and Swarna Rekha showed greatest similarity (0.960) followed by the cultivar Rajat Rekha and Guwahati Single (0.930), whereas, genotypes Vaibhav and Pune Single showed least pair-wise similarity (0.400). The population similarity average value was 0.746.

The accessions were grouped by subjecting the Jaccard's analysis similarity values to UPGMA clustering (Fig. 2). The clusters were identified in such a manner that the similarity within the group was greater but between groups, is less. Thus, the genotypes were grouped into two major clusters. Cluster I included five single petaled genotypes cultivars namely Calcutta Single, Guwahati Single, Pune Single, Saurashtra Single, Rajat Rekha and Prajwal, which is a hybrid. Cluster II had two different sub-clusters. In sub-cluster IIA, there were eleven genotypes all of which were double genotypes except Mexican Single and Shringar.

Table 3. Band statistics generated using RAPD primers in tuberose genotypes.

Sl. No.	Primer	Total no. of amplification product	No. of polymorphic amplification product	Polymorphism (%)	Effective multiplex ratio (EMR)	Diversity index (DI)	Marker index (MI)	Primer resolving power (Rp)
1	OPA 9	10	4	40.0	1.60	0.08	0.13	2.48
2	OPA 13	8	3	37.5	1.12	0.13	0.15	2.16
3	OPB 13	6	4	66.6	2.66	0.11	0.29	1.64
4	OPC 4	11	7	63.6	4.45	0.14	0.62	1.03
5	OPC 8	6	3	50.0	1.50	0.06	0.09	1.42
6	OPC 10	10	5	50.0	2.50	0.16	0.40	1.76
7	OPC12	12	4	33.3	1.33	0.16	0.21	2.22
8	OPC13	18	10	55.5	5.55	0.18	1.00	2.88
9	OPD 3	8	3	37.5	1.12	0.08	0.09	2.32
10	OPD 5	10	1	10.0	1.00	0.05	0.05	0.10
11	OPD 8	14	5	35.0	1.78	0.17	0.30	2.48
12	OPD 12	6	4	66.7	4.16	0.20	0.83	2.68
13	OPD 13	12	5	41.0	2.08	0.09	0.19	0.84
14	OPM 5	10	4	40.0	1.60	0.13	0.20	2.42
15	OPM 10	9	3	33.0	0.99	0.05	0.05	0.65
16	OPM 11	9	3	33.0	0.99	0.08	0.08	2.68
17	OPM 13	9	3	33.0	0.99	0.11	0.11	2.00

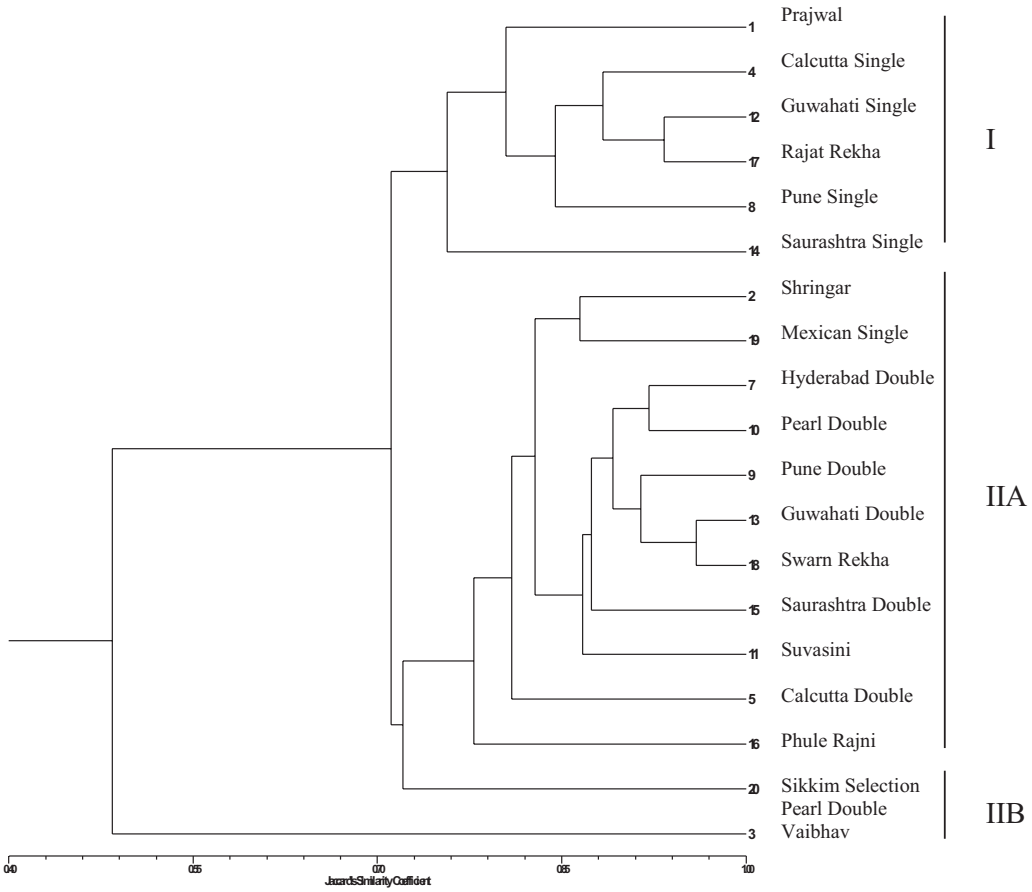

Fig. 2. UPGMA dendrogram of twenty tuberose genotypes

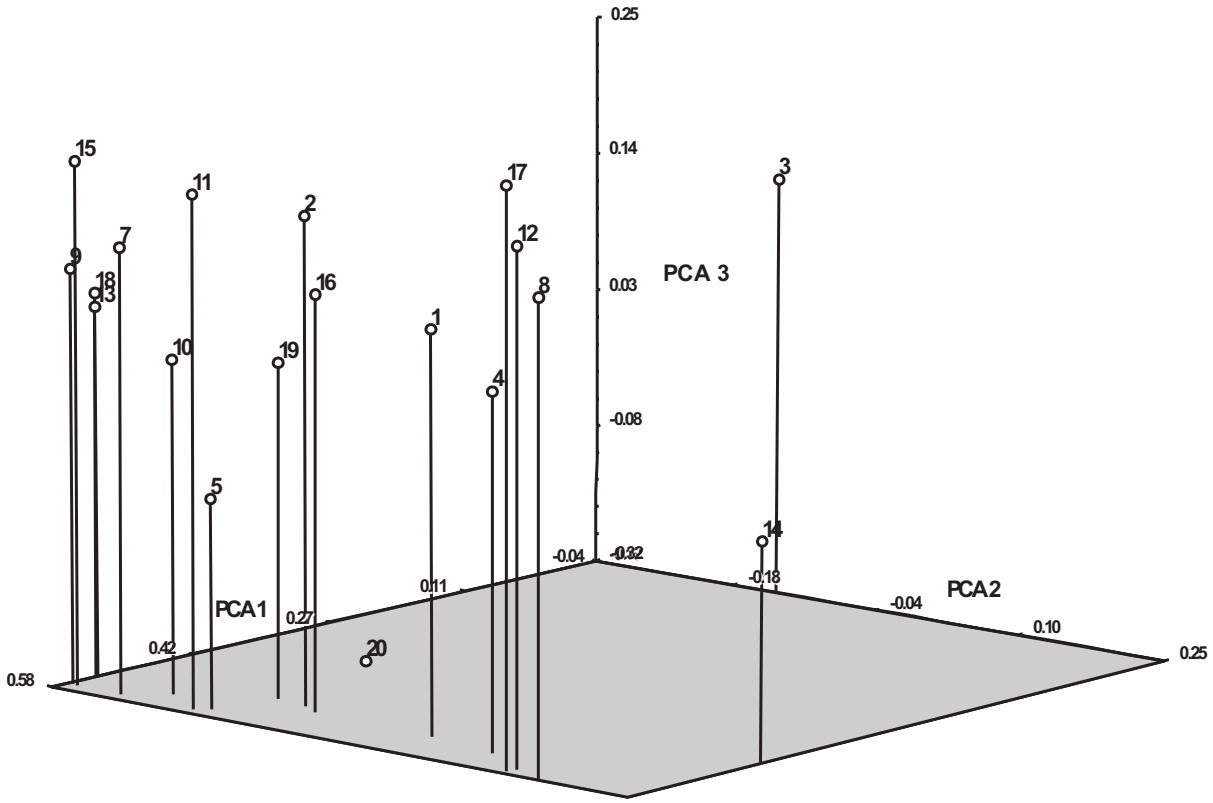

Fig. 3. Principle coordinate analysis of the tuberose genotypes

In sub-cluster IIB, Sikkim Selection was found while Vaibhav was placed separately as different taxa. The clustering of genotypes into different groups could be due to their phenotypic differences like single, double and semi-double types and similarity of the genotypes though common parentage.

The 3D plot of first three principal components is presented in Fig. 3. The components PC1, PC2 and PC3 were used for depicting the three dimensional coordinates that explained 29.94, 19.02 and 8.94% variations. Since, there was only 57.9% variation, 60 principal components were required to explain 100% variation. Three groups were identified in the PCA and among them, Cluster I was identified to be more diverse.

RAPD analysis has proved to be useful in genetic diversity studies [16]. The tuberose genotypes under study showed a moderate degree of variability. It has been reported that in some of the wild plant species lower levels of variations is noted owing to their mating, propagation system and the breeding strategy [17].

Grouping of the genotypes in dendrogram in some cases did not match their phenotypes. For example, Shringar and Mexican Single were single types but grouped in the different clusters. Several reasons may be attributed for these differences, but the most important fact is that the morphological expression is conditioned by stage of the plant, existing agricultural management practices and prevailing environmental conditions. Use of more number of polymorphic markers and accurate phenotyping may reduce it to a great extent.

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