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Genetic diversity analysis and barcoding in tuberose (*Polianthes tuberosa* L.) cultivars using RAPD and ISSR markers

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ABSTRACT

Tuberose is one of the most important bulbous ornamentals grown commercially for loose as well as cut flowers. RAPD and ISSR markers used in the study revealed 53% and 73% polymorphism, respectively, among ten tuberose varieties. Polymorphic Information Content (PIC) and Resolving Power (RP) for RAPD varied from 0.35 – 0.46 and 0.8 – 3.6, respectively, and that for ISSR was 0.36 – 0.49 and 0.91 – 4.55, respectively. The dendrogram (UPGMA), based on Jaccards co-efficient as similarity index for RAPD and ISSR, grouped ten varieties into two major clusters, and, combined RAPD-ISSR cluster analysis formed three major clusters based on their genetic relatedness/variation. PCA revealed that the spatial arrangement of these 10 cultivars was congruent with dendrogram analysis. Mantel's test indicated very good correlation, with r = 0.86 for combination of ISSR and RAPD-ISSR. To facilitate identification of tuberose cultivars, a cultivar identification diagram (CID) was developed in which seven ISSR loci could differentiate all the ten cultivars used in the study. Barcodes were developed for five cultivars released by IIHR using 57 polymorphic loci generated by 11 ISSR primers. The size of these loci ranged from 252bp to 2.2kb. These barcodes can be used as a standard reference source for quick identification of cultivars.

Key words: ISSR, molecular barcode, PCR, RAPD, UPGMA

INTRODUCTION

Tuberose (*Polianthes tuberosa* Linn., Family *Agavaceae*), native to Mexico, is one of the most important bulbous ornamental crops. The genus *Polianthes* comprises 12 species of which nine bear white flowers (Rose, 1903). *P. tuberosa* (2n=30) is the only species used for commercial cultivation in India. The flower spikes are used as an ideal cut-flower which emiting a delightful fragrance and, flower buds are a source of tuberose oil which is one of the most sought-after, expensive perfumery raw materials. The yield of concrete from fresh flower ranges from 0.08 to 0.11 per cent, of which 18 to 23 per cent constitutes the absolute.

Traditionally, morphological and biochemical markers are used for diversity studies and varietal identification, but Caetano *et al* (1991) addressed limitations associated with the morphological and biochemical processes. As in many important horticultural crops, SSRs are not available in tuberose and the cost of developing these is very high. Therefore, during the last decade, RAPD and ISSR markers have been widely exploited by horticulturists as these yield quick results besides being inexpensive. Molecular markers have been successfully used for cultivar identification and diversity studies in several bulbous ornamental plants like *Lilium* species (Yamanishi, 1995), *Alstromeria* (Dobouzet *et al*, 1998), *Heliconia* (Kumar *et al*, 1998) and *Gladiolus* (Takatsu *et al*, 2001; Pathania *et al*, 2001).

Very few studies have been reported so far on molecular characterization of bulbous ornamental crops. Molecular characterization of tuberose cultivars through DNA-based analysis is highly desired, as, there is much confusion in naming genetic material existing in various Indian states. These are commonly referred to as 'single' and 'double' cultivars. Till date, there is only one report on diversity analysis in tuberose using RAPD markers (Sarkar *et al*, 2010).

Indian Institute of Horticultural Research (IIHR) has released five commercial tuberose varieties; Vaibhav, Prajwal, Shringar, Suvasini and Arka Nirantara.

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Characterization of these cultivars has become imperative, since, it is a requisite for provision of plant breeders' rights which is, presently, high on the government's agenda. Further, an understanding of the level of genetic variation among cultivars is crucial to hybridization programs for development of new varieties but is currently lacking. In this study, RAPD and IISR markers were used for genetic analysis of ten tuberose cultivars.

MATERIAL AND METHODS

Plant material and genomic DNA isolation

Ten tuberose varieties maintained at Indian Institute of Horticultural Research, Bangalore, India, were used in this study (Table 1). Total DNA was isolated from the leaf by optimizing modified CTAB method (Kanupriya *et al*, 2011) and integrity of the DNA isolated was determined on 0.8% agarose gel. DNA quantification was carried out using Gene Quant UV Spectrophotometer (GE Health Care Bio-Sciences Ltd., England) and diluted as required.

RAPD analysis

RAPD amplification was performed with a total of 100 RAPD primers of A, B, C, D, H and K series from Operon Technologies, USA. Each 25µl reaction mixture contained 1x reaction buffer (Bioron), 0.12mM each of dNTPs (Genie, Bangalore), 0.15pmol/µl of primer and 1 U of *Taq* polymerase (Genie, Bangalore). PCR amplification was carried out using TECHNE- TC5000 thermocycler with the following profile: initial denaturation at 94^0C for 4 min, followed by 35 cycles of denaturation at 94^0C of 1min each annealing at 35^0C for 30 sec, extension at 72^0C for 1min, and, final extension at 72^0C for 8 min.

Table 1. Tuberose varieties used and their general characteristics

Sl. No.	Variety	Characteristics
1.	Prajwal	Single flowers on tall, stiff spikes; cross of Shringar x Mexican Single (developed by IIHR)
2.	Vaibhav	Double flowers on medium spike, cross of Mexican Single x IIHR-2 (developed by IIHR)
3.	Shringar	Single flowers on a sturdy spike, a cross between Single x Double, (developed by IIHR)
4.	Suvasini	Multi-whorled variety developed from a cross between Single x Double (developed by IIHR)
5.	Arka Nirantara	Hybrid developed by IIHR, single-flower type
6.	Hyderabad Double	More than three rows of corolla segment
7.	Variegated	Single-flowered type, with silvery white streak in the middle of leaf blade
8.	Pearl Double	Flowers pure white, with more than three segments of corolla
9.	Swarna Rekha	Doubled-flower type, with golden-yellow streak along the margin of leaf blade
10.	Mexican Single	Florets bearing a single segment of corolla

ISSR analysis

A total of 108 ISSR primers (University of British Columbia, UK) were screened. PCR amplification was carried out using 1x reaction buffer (Bioron), 0.24 mM each of dNTPs (Genie, Bangalore), 0.2pmol/µl of primer and 1 U of *Taq* polymerase (Genie, Bangalore) in 25µl reaction. PCR amplification was done using TECHNE TC5000 thermocycler, with the following temperature profile: initial denaturation at 94^0C for 4 min, followed by 35 cycles of denaturation at 94^0C of 1min each annealing varying at 49-70 ^{0}C for 45 sec, extension at 72^0C for 75 sec, and, final extension at 72^0C for 8 min.

Amplified products were resolved on 1.5% agarose gel with 1kb ladder (Fermentas, USA) and documented through a gel documentation unit (UVpro, UK) for further analysis.

Statistical analysis

Both RAPD and ISSR gel profiles were scored as discrete variables using '1' for presence and '0' for absence of a band. Polymorphic information content (PIC) was calculated as: $1 - [pi^2 (1- pi)^2]$, where pi is the frequency of i^{th} allele in the dataset. Resolving power (Rp) as sum of band informativeness, Marker Index (MI) and Diversity Index (DI) for both RAPD and ISSR was also calculated. A pair wise matrix of distance between varieties was determined for RAPD, ISSR and RAPD-ISSR data. Cluster analysis was carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method, with DARwin5 software. Principal Component Analysis (PCA) was done using NTSys pc 2.2 software (Rohlf, 2000). Estimates of differences between the dendrograms based on RAPD and ISSR, RAPD and ISSR-RAPD, ISSR and RAPD-ISSR marker analyses were obtained by constructing the relative cophenetic matrices for each marker type. Product-moment correlation (r) based on Mantel Z-value was computed to measure the degree of relationship between similarity index matrices produced by any two-marker systems (Mantel, 1967) using NYSys pc 2.2 software.

Cultivar Identification Diagram (CID) and fingerprinting

CID was constructed using 11 ISSR primers which gave 57 highly reproducible polymorphic bands in ten tuberose varieties, with specific sizes to separate the varieties. Molecular size of each of the fragments was estimated using UVpro software (UVTECH, UK). CID construction is done based on presence (+) and absence (-) of polymorphic loci. Cultivars sharing the same banding pattern were clustered into one sub-group and, subsequently, more markers were employed to distinguish cultivars within each sub-group.

Further, these 11 ISSR markers were used for developing molecular barcodes to fingerprint tuberose varieties. Binary data thus produced is represented as bar for presence and absence of band was kept blank. Molecular barcode was generated by using Microsoft excel programme (Galbacs *et al*, 2009).

RESULTS AND DISCUSSION

RAPD and ISSR analysis

Both RAPD and ISSR markers were used in the study as tools for assessing genetic diversity, to fingerprint and identify different varieties of tuberose. This is the first report on use of RAPD and ISSR markers for diversity analysis and fingerprinting in tuberose.

Of the 100 RAPD primers used 15 gave reproducible results, repeated at least three times for confirmation. Amplification of tuberose DNA of ten varieties with 15 RAPD primers generated 111 fragments, with an average of 7.4 bands per variety. Of these, 59 were polymorphic (53.51%). The fragment size varied from 250bp to 2.0kb. PIC ranged from 0.46 (OPC-20) to 0.35 (OPK-19), Rp 0.8 (OPC-2) to 3.6 (OPC-3), DI 0.13 (OPC-2) to 0.50 (OPA-1), and gene diversity 0.56 (OPB-4) to 0.91 (OPA-1) (Table 2, Fig. 1A). Amplification of tuberose DNA from ten varieties was done with 20 ISSR primers. Of 132 amplified fragments, 95 were polymorphic (73.53%) and the percentage of polymorphism ranged from 100% (UBC-829. 952, 850) to 33.3% (UBC-817); the fragment sizes were 250bp to 2.3kb. PIC ranged from 0.36 (UBC-814) to 0.49 (UBC-836), Rp 0.91 (UBC-880) to 4.55 (UBC-815), DI 0.3 (UBC-901) to 0.56 (UBC-814), MI 0.45 (UBC-880) to 2.82 (UBC-814), and gene diversity 0.68 (UBC-850) to 0.92 (UBC-814) (Table 3, Fig. 1B.).

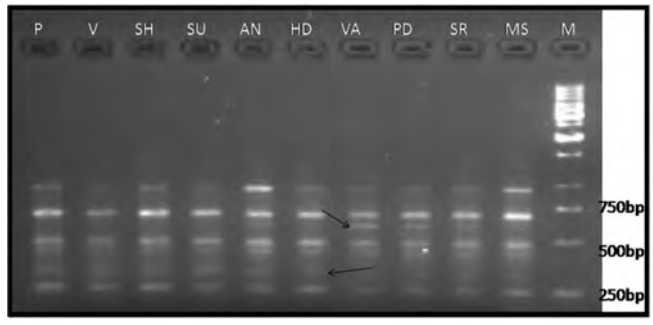
ISSR markers showed a reproducible and polymorphic (73.53%) banding pattern compared to RAPD markers

Table 2. Statistics of banding pattern obtained using 15 RAPD primers

Sl. No.	Primer	No. of bands	N.P.B.	% P.B.	Rp	PIC	DI	MI
1.	OPD-16	11	6	54.54	3.0	0.39	0.38	2.3
2.	OPC-2	8	3	37.5	0.8	0.45	0.13	0.4
3.	OPC-3	10	6	60.0	3.6	0.43	0.31	2.2
4.	OPC-20	6	3	50.0	1.4	0.46	0.23	0.7
5.	OPK-15	6	4	66.66	1.6	0.43	0.20	0.8
6.	OPK-16	7	3	42.85	1.0	0.37	0.37	1.1
7.	OPC-4	10	6	60.0	3.2	0.44	0.33	2.0
8.	OPA-18	5	4	80.0	1.2	0.36	0.35	1.4
9.	OPK-13	6	3	50.0	1.8	0.45	0.30	0.9
10.	OPB14	6	3	50.0	1.4	0.39	0.43	1.3
11.	OPB-4	8	5	62.50	2.0	0.39	0.17	0.69
12.	OPK-19	7	3	42.85	1.6	0.35	0.47	1.4
13.	OPD-3	7	5	71.4	2.4	0.39	0.44	2.2
14.	OPA-1	7	1	14.25	1.0	0.41	0.50	0.5
15.	OPA-2	7	4	57.14	2.4	0.45	0.30	0.6
	Mean	7.4	3.93	53.51	1.89	0.41	0.32	1.23
	Total	111	59					

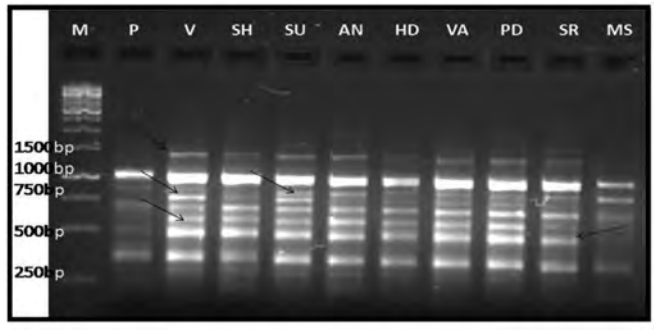
N.P.B - Number of polymorphic bands, % P.B. - Percentage of polymorphic bands; Rp - Resolving Power; PIC - Polymorphic Information Content; DI - Diversity Index; MI - Marker Index

A.



RAPD profile of 10 tuberose varieties on 1.5% agarose using OPK-13
P-Prajwal, V-Vaibhav, SH-Sringar, Su-Suvasini, AN-Arka Nirantara, HD-Hyderabad Double, VA-variegated, PD-Pearl Double, SR-Swarna Rekha, MS - Mexican Single, M - Marker

B.



ISSR profile of 10 tuberose varieties on 1.5% agarose using UBC-867
P- Prajwal, V- Vaibhav, SH- Sringar, Su- Suvasini, AN- Arka Nirantara, HD- Hyderabad Double, VA- variegated, PD- Pearl Double, SR- Swarna Rekha, MS- Mexican Single, M- Marker

Fig .1 Amplification profile of RAPD and ISSR markers in 10 Tuberose varieties

Table 3. Statistics of banding pattern obtained from 20 ISSR primers

Sl. No.	Primer	No. of bands	N.P.B.	% P.B.	Rp	PIC	DI	MI
1.	UBC 813*	8	7	87.5	4.00	0.45	0.36	2.18
2.	UBC 814*	6	5	83.3	3.57	0.36	0.56	2.82
3.	UBC 815*	7	7	100	4.55	0.44	0.41	2.45
4.	UBC 817	6	2	33.3	1.27	0.46	0.32	0.64
5.	UBC 821*	7	5	71.42	1.64	0.42	0.32	1.27
6.	UBC 827	6	5	83.3	2.18	0.39	0.52	1.55
7.	UBC 829*	6	6	100	2.36	0.43	0.30	1.82
8.	UBC 836	8	6	75	3.09	0.49	0.31	1.65
9.	UBC 840*	7	4	57.14	2.18	0.39	0.32	1.91
10.	UBC 841*	6	5	83.3	2.36	0.38	0.48	1.91
11.	UBC 850*	6	6	100	2.91	0.44	0.24	1.45
12.	UBC 852	5	5	100	2.36	0.48	0.24	1.18
13.	UBC 853	4	3	75	1.45	0.45	0.24	0.73
14.	UBC 861	8	6	75	4.36	0.43	0.47	2.36
15.	UBC 864	5	3	60	1.82	0.41	0.45	1.36
16.	UBC 867*	9	6	66.6	2.91	0.45	0.35	1.73
17.	UBC 880	6	1	16.6	0.91	0.44	0.45	0.45
18.	UBC 899*	10	5	50	2.91	0.41	0.45	1.82
19.	UBC 901	6	3	50	1.27	0.41	0.3	0.91
20.	UBC 903*	6	5	83.3	2.73	0.46	0.33	1.64
	Mean	6.6	4.75	72.53	2.54	0.42	0.37	1.59
	Total	132	95					

N.P.B - Number of polymorphic bands, % P.B. - Percentage of polymorphic bands; Rp - Resolving Power; PIC - Polymorphic Information Content; DI - Diversity Index; MI - Marker Index; * Primers used for cultivar identification and fingerprinting

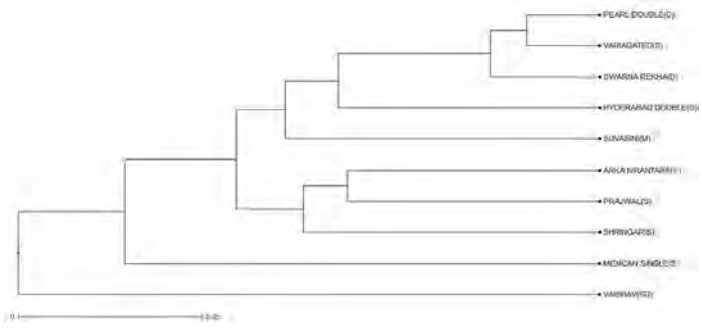
(53.51%). A possible explanation for the difference in resolution of RAPD and ISSR is that these target different sites in the genome (Zietkiewicz *et al*, 1994). PIC, primer resolving power, marker index, diversity index, etc. were found to be greater in ISSR than in RAPD assay. Higher the value in the above analysis, better was the primer for diversity studies. Therefore, ISSR marker system was found to be more effective in diversity analysis among closely related individuals.

Genetic clustering

In UPGMA analysis with RAPD, the genetic distance ranged from 0.040 to 0.293. Ten tuberose varieties grouped into two clusters (Fig. 2A). In the case of ISSR UPGMA analysis, the genetic distance ranged from 0.009 to 0.379, with two clusters. 'Pearl Double' and 'Variegated' were found to be very closely related (genetic distance = 0.009), though these are morphologically distinct in leaf characters (Fig. 2B).

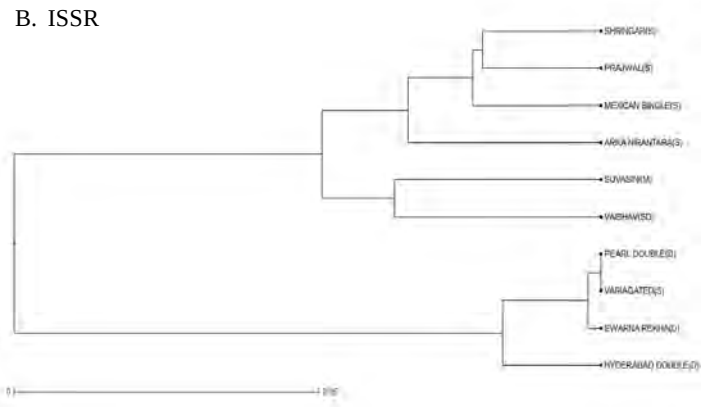
In the case of RAPD+ ISSR primers, UPGMA dendrogram based on a total of 243 bands with 154 polymorphic markers, is presented in Fig. 2C**.** The genetic

A. RAPD



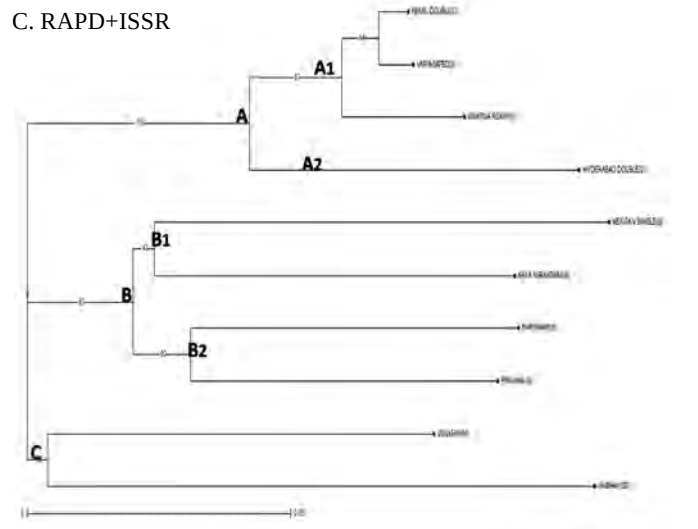
S-Single, D- double, M-Multiwhorl, SD- Single/Double

B. ISSR



S-Single, D- double, M-Multiwhorl, SD- Single/Double

C. RAPD+ISSR

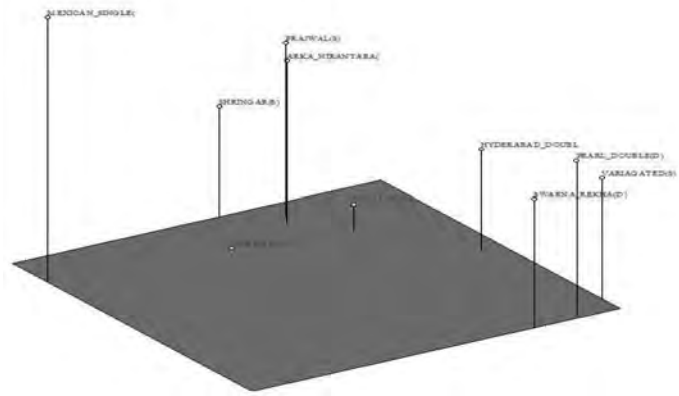


S-Single, D- double, M-Multiwhorl, SD- Single/Double

Fig 2. Dendrograms of ten tuberose varieties generated through UPGMA method of DARWin software using Jaccard's Coefficient as distance matrix

distance was found to range from 0.040 to 0.293. All the 10 varieties grouped into three major clusters. Principal component analysis performed with NTsys pc showed the distribution pattern to be congruent with the dendrogram (Fig. 3).

A. RAPD



B. ISSR

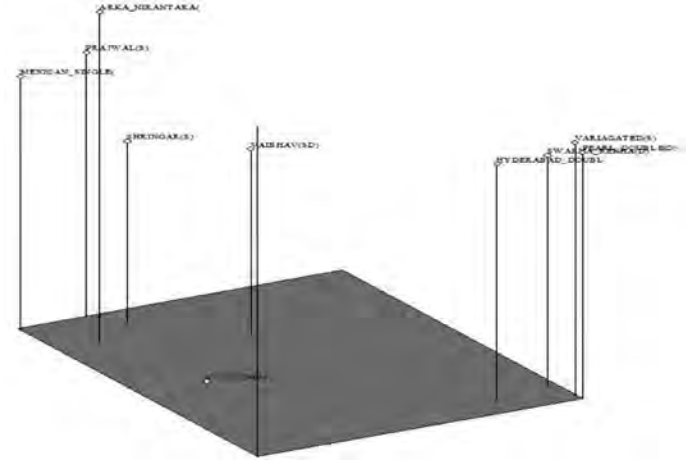


Fig 3. Three-dimensional plots of Principal Component Analysis (PCA) in ten tuberose varieties showing their spatial distribution among three principal components

Due to the differential genome coverage by different marker systems, variation is seen in the three dendrograms generated by RAPD, ISSR and RAPD-ISSR. These dendrograms provided valuable information regarding the similarity of these accessions and their genetic background. Commercial value of tuberose cultivars depends on floral traits. As these varieties showed a distinct clustering pattern based on floral traits, this information may be useful in identifying molecular markers linked to flower morphology. In our study, the flower type (Single, Double, etc.) showed distinct clustering pattern in all the three dendrograms. In some clusters, varieties with Single and Double flowers grouped together. For example, 'Variegated' is the Single type, but it grouped with Double type varieties. Use of greater number of polymorphic markers in such analysis can throw more clarity on clustering pattern. However, compared to the results in RAPD analysis, results in ISSR analyses were more harmonious with morphology. This distinction between the two molecular techniques has previously been indicated in studies with peanut (Raina *et al*, 2001), *Astralagus* (Lin-Kai *et al*, 2009), marijuana (Kayis *et al*, 2010) and *Bacopa monnieri* (Tripathi *et al*, 2012). Weak correlation (Fig. 2A, 2B) was found between RAPD and ISSR ($r = 0.65$, $P < 0.003$), while very good correlation (Fig. 2B, 2C) was found between ISSR and RAPD+ISSR combined marker system ($r = 0.89$, $P < 0.003$) as per Mantel's test. Archak *et al* (2003) also found that genetic similarity matrices based on RAPD and ISSR markers had a low correlation ($r = 0.63$) among cashew accessions.

Cultivar Identification Diagram (CID) and fingerprinting

Compared to phylogenetic trees and fingerprints, CID can provide more comprehensive information for varietal identification. India is one of the important ornamental producing countries and has abundant genetic resources which makes the task of distinguishing plant cultivars or varieties very important.

Owing to a high polymorphism, ISSR data generated was further used in developing cultivar identification diagram and molecular barcodes. Of the 57 polymorphic loci generated by 11 ISSRs, seven loci were used for construction of CID (Fig. 4). Primer UBC-850 (0.6kb) separated ten varieties in to two groups of six (+) and four (-). Primer UBC-867 (0.4kb) differentiated these six varieties again into two groups, based on band presence (Prajwal, Shringar and

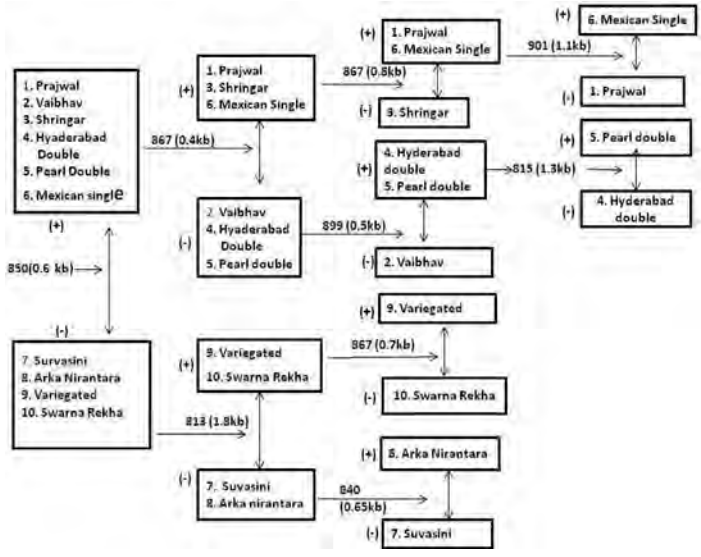


Fig 4. Cultivar identification diagram for 10 tuberose cultivars obtained with seven ISSR loci Note: (+) band present; (-) band absent

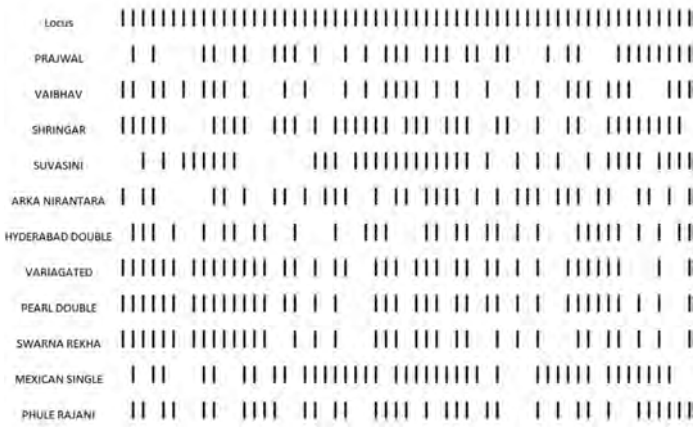


Fig 5. Molecular barcodes that act as unique fingerprints contain 57 different loci generated by 11 ISSR primers in tuberose cultivars.

Mexican Single) and absence (Vaibhav, Hyderabad Double and Pearl Double). The band of 0.8kb of UBC-867 is present in Prajwal and Mexican Single, while, it is absent in Shringar. Further, UBC-901 (1.1kb) differentiated Mexican Single and Prajwal on the basis of the presence and absence of the band, respectively. Primer UBC-899 (0.5kb band) separated Vaibhav (-) from Hyderabad Double and Pearl Double (+); UBC-815, 1.3kb band was present in Pearl Double and absent in Hyderabad Double. Similarly, the second group of four cultivars was differentiated by primer UBC-813 (1.8kb) in Variegated and Swarna Rekha (+); Suvasini and Arka Nirantara (-). Primer UBC-867 (0.7kb) identified variegated (+) and Swarna Rekha (-). A band of size 0.65kb generated by UBC-840 differentiated Arka Nirantara (+) and Suvasini (-). Eventually, all the ten tuberose varieties were successfully differentiated from each other by the combined use of seven ISSR markers.

In molecular barcode, the size of loci ranged from 252bp to 2.208kb. Overall, the chance of identification was found to be $3x10^{-9}$. It can thus be deduced that the primers employed in our study returned a high degree of confidence in identification. The polymorphic 57 loci are represented as 'locus' in the barcode (Fig. 5). From this barcode, any two primers in combination can easily identify all the tuberose varieties, as, these gave unique banding patterns.

RAPD and ISSR techniques have been used for fingerprinting cashew (Archak *et al,* 2003), *Brassica* (Bornet *et al,* 2004) and eggplant (Shiro *et al,* 2008). The results of our study help in identifying 10 tuberose cultivars with much ease and high precision. Seven ISSR loci, in combination, were able to identify all the ten varieties based on the size of polymorphic band. Molecular barcode profile generated with 11 ISSR markers is a visual representation of data, allowing easy detection of genotypic differences. This also helps protect plant breeders' rights and is useful in detecting fidelity when these varieties are multiplied through micropropagation.

Thus, this is the first report of molecular studies in tuberose employing ISSR and RAPD markers for analyzing genetic diversity and fingerprinting. Dendrogram analysis clearly showed that molecular markers used in this study may be linked to the type and number of whorls of petals, i.e., single and double, which can be further developed into specific markers such as SCAR or CAPS. These markers have extensive application in crop improvement through MAS. The molecular barcode generated can be effectively utilized for IPR protection of varieties and used as a standard reference system for varietal identification in tuberose.

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