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Research Paper

Molecular characterization of Tulip (*Tulipa gesneriana*) cultivars utilizing RAPD and ISSR marker system

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ABSTRACT

The genetic variations among ten different tulip (*Tulipa sp*.) genotypes were identified using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) molecular marker system. RAPD analysis was carried out in ten genotypes of tulip with fifteen arbitrary primers, producing 47 loci which were in total 261 amplicons. The pair wise Jaccard's similarity coefficient ranged from 0.31 (between genotype Holland beauty and Avignon) to 0.80 (between Maureen and Holland beauty). The dendrogram based on UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) clearly revealed one individual accession and one cluster, whereas the cluster was subdivided into three sub clusters (Sub cluster-I, Sub cluster-II and Sub cluster-III). ISSR analysis was carried out in the same ten genotypes of tulip with five primers. ISSR primers showing maximum number of polymorphic bands were UBC-14, UBC-15 and UBC-65 (2). The polymorphism percentage shown by different primers ranged from 33.33 to 100. Out of five primers, three primers displayed 100% polymorphism. The results of this study demonstrate the functionality of RAPD and ISSR markers as two effective molecular tools for identification and assessment of genetic relationship within tulip cultivars grown under West Bengal plains.

Key words: DNA isolation, genetic diversity, ISSR, RAPD, Tulip

Tulips (*Tulipa sp*.) are one of the most historically significant and well-known horticultural crop in the world. It is a monocotyledonous plant and also a premier ornamental flowering bulb belonging to family Liliaceae. Tulips are one of the most popular spring flowering plants of all times and ranked third among the top ten flowers sold worldwide (Podwyszynska and Sochacki, 2010). Tulip is a temperate crop and the major center of origin is situated in central Asia (Le Nard and De Hertogh, 1993; Coskuncelebi *et al*., 2008). Being the largest producer of Tulip flowers and bulbs the Netherlands has become the backbone of flower industry. Now Holland is regarded as home of the tulip (Debrowski, 1964) and the other countries with significant global surface are Japan (300 ha), France (293 ha), USA (280 ha) and Poland (200 ha). In India, tulips are successfully grown mainly under the temperate regions of Jammu and Kashmir, Himachal Pradesh, Uttrakhand and similar hilly regions although pre-cooled bulbs are being made to flower during winter in plains. Molecular markers are highly polymorphic and have been successfully used in many bulbous flowering crops (Jingang *et al*., 2008; Kiani *et al*., 2012; Kameswari *et al*., 2014 and Kumar *et al*., 2016). Molecular genetic diversity estimates are extremely useful for accuracy, intellectual property protection particularly in the determination of essential derivation.

RAPD fingerprinting techniques have been used for the identification of horticultural crop varieties, description of cultivar genotypes and for protecting breeder's rights (Williams *et al*., 1990; Camlin, 2001; Debener, 2001b), it has been extensively used to distinguish intraspecific genetic variation in ornamental crops and detection of hybrids and clones (Collins *et al*., 2003; Arus, 2000; Debener, 2001a). ISSR markers were proved to be useful in genetic diversity studies in some flower crops like chrysanthemum (Cai-hong *et al*., 2010; Baliyan *et al*., 2014), lilium (Guo *et al*., 2011; Xi *et al*., 2012; Zhao *et al*., 2014) and Zinnia (Ye *et al*., 2008).

Kameswari and Girwani (2017) used RAPD and ISSR markers to study the genetic relatedness in 37 genotypes of chrysanthemum. Sreedhar *et al*. (2007), during the clonal fidelity analysis of long-term micro-propagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) used RAPD and ISSR markers. An understanding of the level of genetic variation among tulip cultivars is crucial to local breeders involved in hybridization programs for varietal development but is currently lacking. In this study, RAPD and ISSR markers were used to evaluate the extent of genetic variation among ten different tulip cultivars, grown in West Bengal plains.

MATERIALS AND METHODS

Plant material: Ten Tulip cultivars namely Apeldorn (V1), Maureen (V2), Holland beauty (V3), Day Dream (V4), Pursimma Design (V5), Avignon (V6), Roi-du-midi (V7), Clear Water (V8), Coluer cardinal (V9) and Jumbo Beauty (V10) were used in this study. Fresh leaf samples of the mentioned cultivars were collected and used for DNA extraction.

DNA isolation: DNA was isolated from the fresh leaf samples following the modified methods of Sharma *et al.*, 2013**.** Fresh leaf samples (100 mg) were washed in distilled water and rinsed with 80% ethanol. The surface sterilized leaves were kept at -20ºC in refrigerator for 30 min in motar pestle and ground finely with 500 µl extraction buffer and 20 µl of β-mercaptoethanol, 500 µl of extraction buffer was then added and the sample was ground once again. Ground material was transferred to 2 ml centrifuge tube, vortexed and incubated at 37 °C for 30 min and again at 70 °C for 30 min with frequent swirling. Then the mixture was centrifuged at 13000 rpm for 10 min. Supernatant was collected in a new 2 ml microcentifuge tube and equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) was added to that followed by mixing by inversion 50-60 times. The mixture was again centrifuged at 13000 rpm for 10 minutes and the supernatant was poured into a new 2 ml microcentifuge tube. 200 µl of 4% PEG solution was then added and mixed thoroughly, the mixture was incubated at 0°C. After 20 min the mixture was again centrifuged at 13000 rpm for 10 min. The supernatant containing DNA was collected in a separate 2 ml microcentifuge tube followed by addition of pre-cooled isopropanol (65% volume of supernatant). It was then mixed thoroughly and waited for 5 min at room temperature. DNA was precipitated after centrifugation at 13000 rpm. The supernatant was discarded and 500 µl wash solution was added in the 2 ml microcentifuge tube. After centrifugation the DNA pellet was collected and air dried until thealcoholic smell completely disappeared. DNA pellets were suspended in 100 µl of TE buffer. Suspended DNA was treated with 1 µl of RNAse (1.43 µg/µl, Sigma) and incubated at 37°C for 30-40 min. The extracted DNA samples were stored at -20°C until use.

DNA analysis: The quality of extracted DNA was analyzed using agarose gel electrophoresis (0.8%), followed by ethidium bromide staining. The purity of the DNA was estimated by spectrophotometry using A_{260}/A_{280} ratio, and the yield was estimated by measuring absorbance at 260 nm.

Detection of polymorphism utilizing Random Amplified Polymorphic DNA (RAPD) marker system: A set of fifteen screened random decamer oligonucleotide primers (OPN-07, OPN-09, OPN-10, OPN-14, OPO-01, OPO-03, OPO-07, OPO-18, OPO-19, OPP-03, OPP-14, OPP-15, OPP-16, OPQ-04, OPQ-06) were used for RAPD analysis. Each 25 µl of PCR mixture consisted of 20 ng of template DNA, 100 µM each deoxynucleotide triphosphate, 20ng of decanucleotide primers (Sigma), 1.5 mM $MgCl_2$, 1X Taq buffer (supplied with the Taq Polymerase enzyme), 1U of Taq DNA polymerase (Xcelris). Amplifications were performed in Veriti Thermal Cycler (Applied Biosystems). The PCR mixtures were heated at an initial step of 94 °C for 3 min and then subjected to 35 cycles of following programme: 94 °C for 30 sec, 40 °C for 45 sec, 72 °C for 1 min. After the last cycle, the temperature was maintained at 72 °C for 5 min. Amplified products were resolved on a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (QUANTUM-ST4, LED's bar Epi-illumination, France). Clear bands were revealed and were scored for their presence (1) or absence (0) following the methods of Williams *et al*., 1990. All profiles were reproducible and gave clear and easy to score bands.

Detection of polymorphism utilizing Inter Simple Sequence Repeat (ISSR) marker system (*Following the method of Son et al.*, 2013):

Thermocycler conditions: The PCR thermocycling conditions that yielded optimum PCR amplification were as follows.

1. Denaturation at 94° °C for 2 minutes.

2. Thirty five cycles of 1 minute denaturation at 94 °C, 1 minute annealing at 35 °C and 2 minutes primer extension at 72 °C.
3. One final step of extension at 72 °C for 5 minutes.

Optimization of PCR conditions: Eight different DNA concentrations (5, 10, 15, 20, 25, 30, 35, 40 ng) were tried. The best result was obtained with 10ng and hence, a DNA concentration of 10ng per reaction was used in this study. After testing different enzyme concentration (0, 0.25, 0.5, 0.75, 1, 1.25, 1.50 U). 0.5 U of Taq DNA polymerase was found to yield optimum amplification product. A concentration of 1.5 mM $MgCI_2$ per reaction gave the best results among the different concentration (0.5, 1.0, 1.5, 2.0 and 2.5 mM) tried. Based on these observations, the optimum concentration of PCR components for tulip ISSR (for 25 µl reaction mixture) is presented below (Table 1 and Table 2).

Table 1: Composition of PCR mixture for ISSR analysis in tulip

Component	Concentration
Template DNA	10 ng
PCR assay buffer	1X
$MgCI_2$	1.5 mM
dNTPs (dATP, dGTP, dCTP and dTTP)	200 µM
Taq DNA Polymerase	05. U
Primer	O.4 µM

Table 2: Details of the used ISSR primers

S. no.	Primer Id	Sequence	Tm (ºC)	Ta (ºC)
1	UBC-14	$(CT)_8A$	41.4	40
2	UBC-15	$(CT)_8G$	45.0	40
3	UBC-16	$(CA)_8$	49.9	45
4	UBC-20	$(GT)_8C$	50.4	45
5	UBC-65	$(CCG)_6$	89.3	55

RESULTS AND DISCUSSION

Analysis of RAPD carried out in ten genotypes of tulip with fifteen arbitrary primers produced 47 loci which were in total 261 amplicons. Out of the 47 loci produced, 33 were polymorphic, amounting to a total polymorphism percentage of 71.67. Six primers out of the 15 analyzed, produced more than 75% polymorphism. The primers OPP-15, OPN-07 and OPO-07 showed 100% polymorphism in the population. The PIC (Polymorphic Information Content) values ranged from 0.34 (OPQ-04) to 0.96 (OPN-14) with an average value of 0.64, indicating hyper-variability among the accessions studied. NTB (Number of Total Band) values for all the primers ranged between 2 to 4 with an average 3.13. The average NPB (Number of Polymorphic Band) value for all the RAPD primers was 2.20 and ranged from 1 to 3. The primer OPN-14 showed the lowest NPB (Number of Polymorphic Band) value (1.0). The average Polymorphism Percentage (PP) value for the RAPD primers was 71.67 and ranged from 50 to 100.

Correlations between the efficiency parameters were non-significant. The Number of Total Bands showed the negative correlation with the Polymorphism Percentage. However, the PIC value showed positive relation with all other parameters. This result indicates that all the efficiency parameters were not equally important and selection of primer on the basis of PIC value will be effective for future study. The genotypic data generated through RAPD profiling of 10 tulip accessions were used to study genetic diversity or interrelationship. The pair wise Jaccard's similarity coefficient ranged from 0.31 (between Holland beauty and Avignon) to 0.80 (between Maureen and Holland beauty). The dendrogram based on UPGMA clearly revealed one individual accession and one cluster, whereas the cluster was subdivided into three sub clusters (Sub

cluster-I, Sub cluster-II and Sub cluster-III) (Fig.1). The Sub cluster-I was the smallest having only one accession whereas Sub cluster-III was the largest with 5 (50 %) accessions followed by cluster II with 4 (40%) accessions. In order to validate efficiency of primers in distinguishing genetic diversity, PCA was performed, which supported the results of cluster analysis using pair wise Jaccard's similarity coefficient (Fig.2). The genetic distances predicted on the basis of 15 primers presented a vast range citing that tulip genotypes personify genetically diverse populations. Selection for breeding programs can be done from the obtained clustering patterns and the genetic relationship.

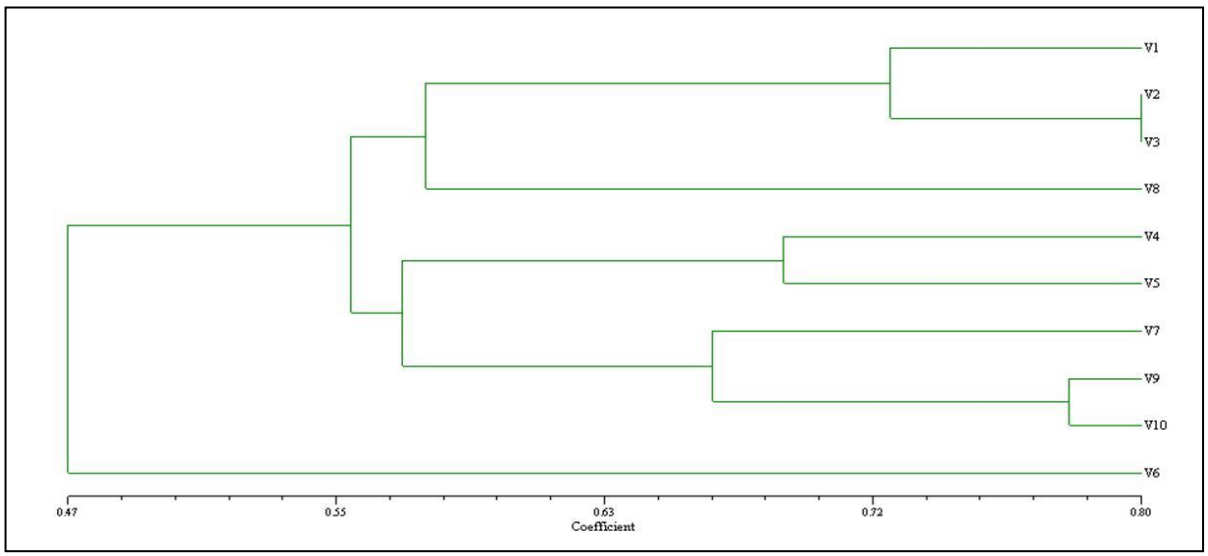

Fig.1: Dendrogram revealed by RAPD profiling

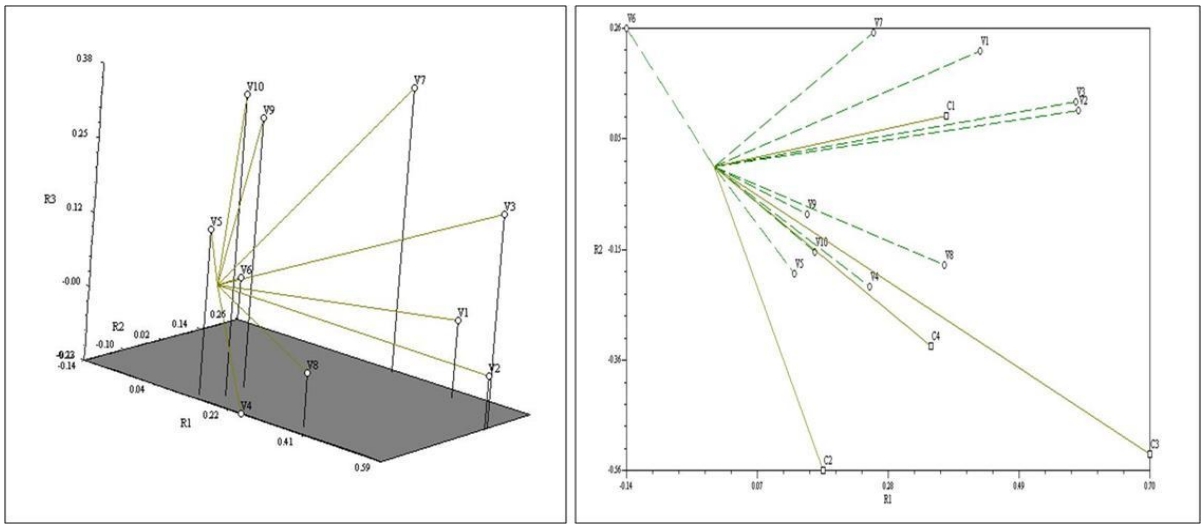

Fig. 2: PCA analysis using RAPD markers technology showing distinct clustering pattern

In the other hand, the ISSR primers showing maximum number of polymorphic bands were UBC-14, UBC-15 and UBC-65 (2). The polymorphism percentage shown by different primers ranged from 33.33 to 100. Out of five primers, three primers displayed 100% polymorphism. The primer resolving power (RP), marker Index (MI) and effective multiplex ratio (EMR) were found to be highest for the primer UBC-15, while lowest was for the primer UBC-20 (Table 3). Maximum polymorphic information content (PIC) was observed for the primer UBC-20 (0.48) and its diversity index (DI) was also comparatively higher (0.64). Primer UBC-15 was found to have highest diversity index (0.93) with comparatively higher value of PIC (0.34). From the above comparisons, the primers UBC-14, UBC-15 and UBC-20 were identified as efficient primers. As evident from Table 3, the primers with maximum number of polymorphic loci had comparatively high amount of effective multiplex ratio and marker index with high resolving power.

Table 3: Various parameters related to efficiency of 5 primers for ISSR analysis

Primer Id	NTB	NPB	PP	PIC	EMR	DI	RP	MI
UBC-14	2.00	2.00	100.00	0.40	1.45	0.60	1.20	0.87
UBC-15	3.00	2.00	66.67	0.34	1.45	0.93	1.47	1.35
UBC-16	3.00	1.00	33.33	0.26	0.73	0.83	1.00	0.60
UBC-20	1.00	1.00	100.00	0.48	0.73	0.64	0.80	0.46
UBC-65	2.00	2.00	100.00	0.42	1.45	0.51	1.20	0.74

Molecular diversity analysis: Genetic relationships between the tulip accessions were determined on the basis of Jaccard's pairwise similarity coefficients. The similarity values obtained between pairs of accessions are presented in Table 4 Cultivar pair, Coluer cardinal (V9) and Jumbo Beauty (V10) showed the greatest similarity (1.00) followed by cultivar Day Dream (V4) and Pursimma Design (V5) (0.91). Cultivar Maureen (V2) showed the least pairwise similarity with all other cultivars. However, it had a greater similarity (0.73) with Roi-du-midi (V7). The mean similarity for all pairs of comparisons was 0.72.

Table 4: Similarity Matrix Value of ISSR analysis

Genotypes	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
V1	1.00									
V2	0.73	1.00								
V3	0.82	0.55	1.00							
V4	0.64	0.36	0.64	1.00						
V5	0.73	0.45	0.73	0.91	1.00					
V6	0.64	0.55	0.45	0.64	0.73	1.00				
V7	0.64	0.73	0.45	0.64	0.73	0.82	1.00			
V8	0.64	0.55	0.45	0.45	0.55	0.64	0.64	1.00		
V9	0.45	0.55	0.27	0.45	0.55	0.64	0.64	0.82	1.00	
V10	0.45	0.55	0.27	0.45	0.55	0.64	0.64	0.82	1.00	1.00

Cluster analysis: The accessions were grouped by subjecting the Jaccard's similarity values to UPGMA clustering (Fig.3). The clusters were identified in such a manner that the Similarity within group was greater but between the groups it should be less. Thus, at the point of population average of 0.72 similarity, the clusters were designated and it resulted in identification of two major clusters. Cluster I included 4 cultivars. Cluster II had 6 accessions.

Boot strapping: The reliability of the nodes of UPGMA tree was tested by bootstrap analysis using 1000 pseudo replicates. These proportions were used to assess the reliability of individual branches in the optimal tree. In this analysis, all higher branches of UPGMA dendrogram were supported by more than 50% values.

PCR based markers are gaining much popularity nowadays to assess genetic diversity among horticultural crops. Diversity analysis of any crop species is first step towards crop improvement. The results of present study assess diversity among 10 accessions of tulip through ISSR markers. Kiani *et al.* (2012) also demonstrated that the ISSR technique is very effective in determining the genetic diversity of species within the genus *Tulipa*. Furthermore, ISSR markers have another advantage in tulip: there are no strict requirements for the quality of extracted DNA, which is particularly important for liliaceous family because of their high polysaccharide content. Therefore, ISSR genotyping is a suitable method for studying the genetic diversity of tulips. The differences in genetic parameters indicated that tulip cultivars had rich genetic diversity. The cluster based on

ISSR polymorphism indicates that ISSR markers can be used to quick and accurate identification of tulip cultivars and determination of the exact type of an unknown tulip variety.

In conclusion, from the present study, it has been observed that RAPD and ISSR maker systems are simple and quick method for estimating the genetic diversity analysis in tulip. Based on present investigation a good amount of genetic difference was noticed among all the ten accessions through RAPD and ISSR molecular markers. Knowledge on genetic diversity will help in the efficient management of tulip germplasm by breeders.

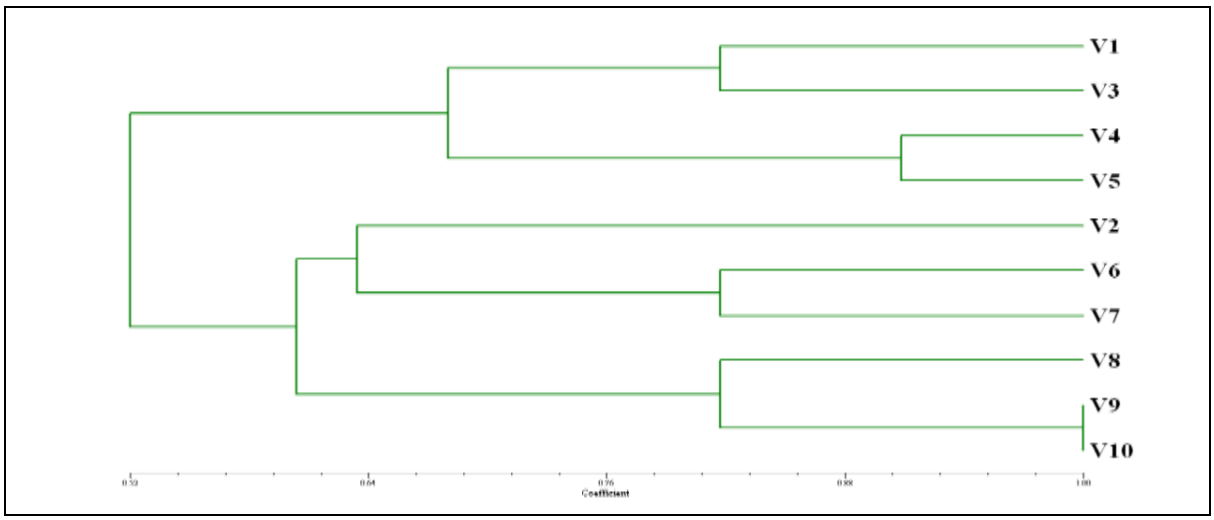

Fig. 3: UPGMA cluster analysis of ISSR data generated by five primer combinations for 10 tulip genotypes depicting genetic diversity. [Scale depicted represents genetic estimates]

CONCLUSION

RAPD fingerprinting techniques and ISSR markers have been used for the identification of horticultural crop varieties, description of cultivar, genetic diversity studies in various flower crops. Tulip being an important flower crop need proper understanding of the level of genetic variation among tulip cultivars for local breeders involved in hybridization programs for varietal development. The study was intended used to evaluate the extent of genetic variation among ten different tulip cultivars by use of RAPD and ISSR markers. And, this study demonstrated the functionality of RAPD and ISSR markers as two effective molecular tools for identification and assessment of genetic relationship within tulip cultivars.

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