Establishing Distinctiveness in Local Maize (*Zea mays* L.) Varieties using RAPD Marker as Additional Descriptor for Protection under PPV&FR Act

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(Received: 26 February 2012; Revised: 13 September 2012; Accepted: 17 September 2012)

The molecular identification profiles were established in the present work for 24 local maize varieties with 10 RAPD markers to incorporate the fingerprint, as complementary information to the standard registration data on DUS morphological descriptors. It resulted in 172 informative RAPD loci. UPGMA analysis of RAPD marker loci could discriminate all the varieties except Khusalpur Local-4 and Nunawala Local-5. These primers could establish unique molecular identification profiles (MIPs) for a total of 14 varieties. Mean polymorphic information content (PIC), average expected gene diversity, average resolving power (Rp) and diversity index (DI) of RAPD markers used were high which reflected that RAPD marker is an efficient tool to establish distinctiveness among the present set of experimental material. Principal component analysis (PCA) of RAPD data supported UPGMA clustering results. Thus, the present investigation reflects that molecular markers are new possibilities as additional descriptor to characterize the plant cultivars for registration purposes under PPV&FR Act.

Key Words: Diversity index, Maize, Molecular identification profile, PPV&FR Act, RAPD

Introduction

Maize (*Zea mays* L.) an allogamous crop is an important cereal used as food for human consumption, feed for animal and poultry and as an important source of edible oil and starch. In India during 2010, it occupied an area of 7.18 million hectares with production of 14.06 million tonnes and productivity of 1958.22 kg/ha (http://faostat.fao.org). India has an immense wealth of maize varieties growing in all the different agroclimatic regions. There is a need in the country to protect such a vast variability present in the species, which is conserved by farmers as local indigenous varieties. The matter of protection of varieties started only after an international body UPOV was established in Paris in 1961 and it entered into force in 1968. UPOV aimed to ensure protection of varieties by the grant of an exclusive right on the protected new plant variety on the basis of a set of principles (Dutfield, 2001). On the UPOV pattern India has enacted a *sui generis* form of protection as Protection of Plant Varieties and Farmers' Rights Act (PPV&FR Act), 2001. Under PPV&FR Act a variety must fulfill the criteria of distinctiveness, uniformity, stability (DUS) and novelty (if newly developed) so as to get protection under the Act (Anonymous, 2001). As per DUS guidelines of PPV&FR Act Authority there are 31 morpho-physiological DUS descriptors for maize which are species-specific and recommended procedures for conducting DUS trials (Anonymous, 2007). Plant morphological descriptors have been the universally undisputed traits applied for DUS testing of crop varieties but serious problems may arise in future for establishing distinctiveness of variety only on morpho-physiological DUS descriptors especially in closely related local cultivars (Patra *et al*., 2010). These morphological descriptors suffer from many drawbacks, such as influence of environment on trait expression, limited in number and increasing number of candidate varieties with decreased variability which enforces to look for alternatives (Joshi *et al*., 2009). Biochemical markers *viz.* isozymes and storage proteins have been mentioned to complement morphological markers under UPOV guidelines (Anonymous, 1999). But results obtained from them may not be up to the mark by the general consideration since only a minor portion of the genome is represented by these markers (Stuber *et al*., 1988). Genetic diversity studies on maize using traditional morphological and biochemical markers are common and routinely used. However, with the availability of large number of polymorphic molecular markers *viz.* RAPD, RFLP and SSRs created interest in their use for varietal identification (Krevich *et al*., 1992; Mackill, 1995; Mc Gregor *et al*., 2000). Among the diverse range of molecular marker techniques available for evaluating genetic diversity, RAPDs are well known for their potentially high information content and versatility

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(Pejic *et al*., 1998; Singh *et al*., 2009). RAPD analysis in aromatic and basmati rice varieties revealed high degree of polymorphism and were shown to complement DUS morphological descriptors in establishing distinctiveness of varieties (Patra *et al*., 2010; Joshi *et al*., 2010). In the present study PCR based molecular marker RAPD was used as additional descriptor to evaluate the genetic diversity and to develop DNA profiles of 24 local maize varieties representing different geographical locations of Uttarakhand. DNA fingerprinting has rendered genotype characterization highly efficient enabling reliable distinction of even closely related accessions. This study was conducted in anticipation, if morpho-physiological DUS descriptors are not able to discriminate closely related varieties, then molecular markers can be considered as additional descriptors for establishing the distinctiveness.

Material and Methods

Plant Material

A total of 24 maize varieties were selected; 22 varieties from wide range of geographical locations of Uttarakhand and two reference varieties from Karnal and Pantnagar were selected. The details of the cultivars are given in Table 1.

Table 1. Details of maize varieties studied with their origin

Sl. No.	Genotype	Areas of cultivation
1	Dharap Local-4	Nainital
2	Kirtinagar Local-4	Dehradun
3	Purvi Khera Local-1	Haldwani
4	Paschim Khera Local-2	Haldwani
5	Manacot	Garhwal
6	Khusalpur Local-1	Garhwal
7	Khusalpur Local-3	Garhwal
8	Khusalpur Local-4	Garhwal
9	Khusalpur Local-6	Garhwal
10	Nunawala Local-1	Haridwar
11	Nunawala Local-5	Haridwar
12	Nunawala Local-6	Haridwar
13	Doiwala Local-2	Dehradun
14	Bailparow Local-4	Nainital
15	Bhadarabad Local-4	Haridwar
16	Bhadarabad Local-5	Haridwar
17	Bhadarabad Local-8	Haridwar
18	Bagauli Local-1	Haridwar
19	Bishanpur Local-2	Haldwani
20	Khera Local	Haldwani
21	Chamoli Local	Dehradun
22	Quarali Local-1	Garhwal
23	Pragati	Pantnagar
24	HQPM 1	Karnal

Molecular Characterization

The genomic DNA was extracted from 14-day-old etiolated seedlings following CTAB method (Doyle and Doyle, 1990) with minor modifications. The quantification of DNA in RNA free sample was done using a UV visible spectrophotometer (ELICO Ltd.). The quality and quantity of DNA was checked by 1% agarose gel electrophoresis using standard containing 100 ng/µl genomic DNA. RAPD analysis was performed in a 0.2 ml reaction tube in volume of 25 µl containing 10 X Assay Buffer, 0.5 unit of *Taq* DNA polymerase, 200 µm each of dNTPs, 50 ng/µl reaction of random primer and 50 ng of template DNA (standardized). Molecular characterization was conducted with 10 random primers as shown in Table 2. PCR was performed in 'Eppendorf Thermocycler' by initial denaturation at 94°C for 5 min followed by 39 cycles of denaturation at 94°C for one min, annealing at 32°C for one min, extension at 72°C for two min and final elongation at 72°C for 7 min. The PCR products were electrophoresed on 1.2% agarose gel, prepared in 1X TBE buffer containing 0.5 µg /ml of the ethidium bromide at 80V for 3h with cooling. The gel was photographed under UV transilluminator. The PCR reaction was repeated twice for each primer. The non-reproducible bands, weak or smeared bands were not counted and also not included in the comparative analysis. Profiles for each cultivar and marker system were constructed by scoring in a binary matrix as "0" and "1" for the absence and presence of fragments respectively and the final data sets included both polymorphic and monomorphic fragments. Cluster analysis was performed using SHAN module of the NTSYS-pc (Numerical Taxonomy System, version 2.0) (Rohlf, 2002). Similarities between varieties were estimated using the SIMQUAL program to calculate the Jaccard's coefficient by Unweighted Pair Group Method on Arithmetic Averages (UPGMA), a common estimator of genetic identity (Jaccards, 1908). Strength of the clusters was analyzed by bootstrap values calculated by using 1000 sampling with Win Boot Software (Yap *et al*., 1995). It performs analysis of binary data to determine the confidence limits of UPGMA-based dendrogram. PIC that provides an estimate of the discriminatory power of a locus or loci, by taking into account, not only the number of alleles that are expressed, but also relative

Table 2. Details of RAPD primers used for the molecular characterization of 24 maize varieties

Sl.No.	Primer code	Primer sequence	Amplified product (Kb)	Total bands	Mono-morphic bands	Poly-morphic	% Polymorphism	PIC	Average expected gene diversity (Hi)	Resolving power (Rp)
1	P1	GTAGCACTCC	0.2 Kb- 2.0 Kb	17	5	12	70.6%	0.5	0.1	16.83
2	P2	TCGGCACGCA	0.3 Kb- 1.7 Kb	22	1	21	95.5%	0.4	0.3	19.58
3	P3	GTGTCTCAGG	0.3 Kb- 2.0 Kb	16	0	16	100%	0.7	0.2	22.08
4	P4	GTCCATGCCA	0.3 Kb- 2.0 Kb	16	1	15	93.8%	0.4	0.3	14.33
5	P5	ACATCGCCCA	0.3 Kb- 4.0 Kb	18	10	8	44.5%	0.3	0.1	9.00
6	P6	CCAGCCGAAC	0.3 Kb- 1.7 Kb	17	2	15	88.2%	0.5	0.3	16.00
7	P7	GGAAGCCAAC	0.2 Kb- 3.0 Kb	18	5	13	72.2%	0.4	0.2	15.92
8	P8	CCAAGCTGCC	0.3 Kb- 3.0 Kb	11	4	7	63.7%	0.5	0.1	10.92
9	P9	TGCGGCTGAG	0.3 Kb- 2.5 Kb	16	9	7	43.8%	0.2	0.1	5.17
10	P10	GACGGATCAG	0.4 Kb- 2.5 Kb	21	4	17	81%	0.4	0.2	17.58
			Average	**17.2**	**4.1**	**13.1**	**75.33**	**0.43**	**0.19**	**14.8**

frequencies of those alleles was calculated using the formula PIC = $1-\sum p_i^2$, where p_i is the frequency of the i^{th} allele. The Rp to distinguish among the studied genotypes of a primer was calculated using the formula Rp = Σ Ib where Ib is band informativeness. It takes the value of: 1 – [2 × (0.5 – p)], p being the proportion of the 24 maize varieties containing the band (Torre *et al*., 2006). The Jaccard's similarity matrix was subjected to principal component analysis (PCA) (Yadav *et al*., 2010). The first three principal components were used for 3-dimensional plotting, amongst each other using module PROJ and MXPLOT of NTSYSpc. This method makes use of multi-dimensional solution of the observed relationships based on the genetic distance.

Results and Discussion

The molecular markers play a relevant role in determining cultivar diversity and hence leading to intellectual property rights protection. The accurate description of maize varieties and distinctiveness is crucial for registration under PPV&FR Act. The identity/profile of a variety is to be established by using a set of morphological characteristics prescribed in the DUS test guidelines on maize. On the basis of DUS morphological characters only 11 varieties could be discriminated. Thus, morpho-physiological DUS descriptors alone were not sufficient for establishing the distinctiveness especially in closely related varieties or similar indigenous local varieties grown in a particular niche. Hence, in the present study molecular markers were considered for establishing the distinctiveness of maize varieties.

RAPD Analysis

RAPD marker analysis was conducted on DNA extracted from the 24 varieties using 10 oligonucleotide random primers. The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), PIC, Rp and DI are shown in Table 2. All the primers amplified 172 RAPD loci (average of 17.2 bands per primer) across the 24 genotypes studied, out of which 131 loci were polymorphic and 41 were monomorphic. Amplification products ranged in size from 4.0 kb (by the primer P-5) to 0.2 kb (by the primers P-1 and P-7). Maximum number of 22 amplification products were obtained with the primer P-2, followed by 21 products with primer P-10 while minimum number of 11 RAPD loci were generated with primer P-8. One primer produced 100% polymorphism and 2 primers showed more than 90% polymorphism. However other primers together accounted for 75.3% polymorphism. The unique MIPs were created from different RAPD primers, which could identify the fourteen varieties. Out of 14 varieties, maximum number of 4 unique bands were observed in Bhadarabad Local-5 and Bishanpur Local-2 while Kirtinagar Local-4, Purvi Khera Local-1 and Khera Local showed 3 unique bands in each variety. Dharap Local-4, Nunawala Local-6, Doiwala Local-2 and Bagauli Local-1 showed 2 unique bands in each variety while Paschim Khera Local-2, Khusalpur Local-6, Bailparow Local-4, Chamoli Local and HQPM 1 showed 1 unique band in each variety. The unique band appear in Doiwala Local-2 by P-5 is shown in Fig. 2. However, for ten varieties no unique MIPs were obtained by any of the primers studied. The size and number of these exclusive or genotypic specific bands amplified in the mentioned varieties are presented in Table 3. These unique bands generated in mentioned varieties reveal distinctiveness of varieties. The PIC value ranged from 0.2 for primer P-9

Table 3. Number and size of genotype specific bands amplified by RAPD markers in 24 maize varieties

Sl. No.	Varieties	RAPD		
		Primer code	No. of exclusive loci	Size of exclusive loci
1	Dharap Local-4	P3	1	0.4 Kb
		P10	1	0.45 Kb
2	Kirtinagar Local-4	P2	1	1.4 Kb
		P7	1	0.48 Kb
		P8	1	0.3 Kb
3	Purvi Khera Local-1	P1	1	0.55 Kb
		P3	1	0.70 Kb
		P10	1	0.65 Kb
4	Paschim Khera Local-2	P10	1	0.7 Kb
5	Manacot	-	-	-
6	Khusalpur Local-1	-	-	-
7	Khusalpur Local-3	-	-	-
8	Khusalpur Local-4	-	-	-
9	Khusalpur Local-6	P6	1	1.0 Kb
10	Nunawala Local-1	-	-	-
11	Nunawala Local-5	-	-	-
12	Nunawala Local-6	P9	1	0.35 Kb
		P10	1	0.95 Kb
13	Doiwala Local-2	P5	1	0.5 Kb
		P10	1	1.7 Kb
14	Bailparow Local-4	P8	1	0.45 Kb
15	Bhadarabad Local-4	-	-	-
16	Bhadarabad Local-5	P1	1	0.55 Kb
		P1	1	0.40 Kb
		P3	1	1.8 Kb
		P7	1	1.5 Kb
17	Bhadarabad Local-8	-	-	-
18	Bagauli Local-1	P1	1	1.5 Kb
		P7	1	0.5 Kb
19	Bishanpur Local-2	P1	1	0.65 Kb
		P4	1	0.35 Kb
		P7	1	0.9 Kb
		P8	1	0.7 Kb
20	Khera Local	P2	1	1.1 Kb
		P2	1	0.52 Kb
		P3	1	0.90 Kb
21	Chamoli Local	P6	1	1.1 Kb
22	Quarali Local- 1	-	-	-
23	Pragati	-	-	-
24	HQPM 1	P2	1	0.3 Kb

to 0.7 for primer P-3 with an average of 0.43 for all the ten primers which shows the ability of different primers to discriminate among the maize cultivars. 'Prevost and Wilkinson' reported the Rp as the capacity of a given primer to discriminate among different genotypes (Prevost A., 1999). Seven RAPD primers *viz*. P-1, P-2, P-3, P-4, P-6, P-7 and P-10 having high Rp of 16.83, 19.58, 22.08, 14.33, 16, 15.92 and 17.58, respectively, were able to discriminate majority of the varieties. The 3D depiction of PCA results are shown in Fig. 1. According to PCA results, the 24 varieties were well separated and RAPD data also well supported their UPGMA clustering. PC1, PC2 and PC3 accounted for 11.43%, 7.65% and 6.3% of the variation, respectively and the cumulative variation of these three principal components was 25.38%.

Genetic Relationships of Varieties

The Jaccard's coefficients for the genetic similarities among the 24 varieties are presented in Table 4. The similarity coefficient values varied from 0.57 between Purvi Khera Local-1 and Doiwala Local-2 to 0.85 between Khusalpur Local-4 and Nunawala Local-5. The

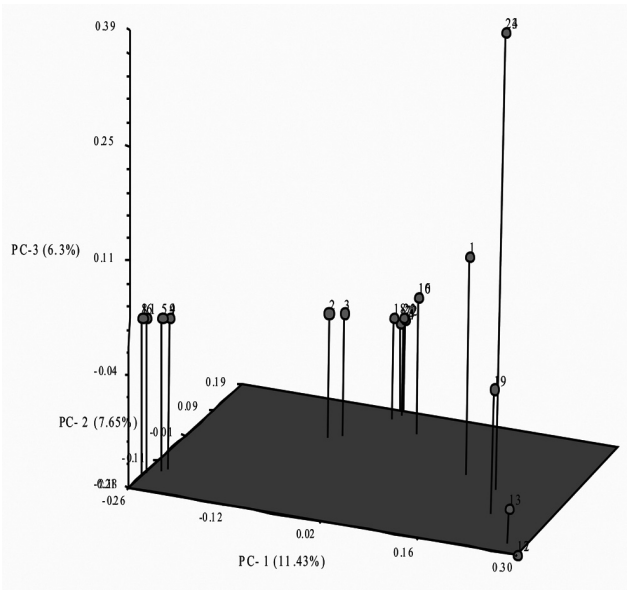

Fig. 1. Three dimensional plot based on Principal Components 1, 2 and 3 of 24 maize varieties using RAPD marker. The three axes represent the first 3 principal components (PC) (1-24: Names of varieties as mentioned in Table 1)

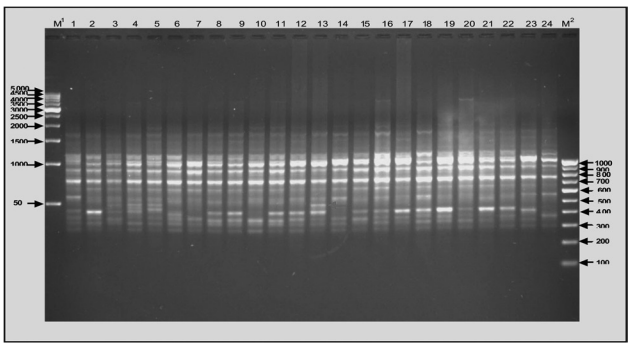

Fig. 2. Molecular diversity generated among the 24 maize varieties by RAPD primer P-5. (1-24: Names of varieties as mentioned in Table 1)

Table 4. Genetic similarity matrix of 24 maize varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.00																							
2	0.70	1.00																						
3	0.66	0.74	1.00																					
4	0.68	0.74	0.72	1.00																				
5	0.71	0.71	0.70	0.82	1.00																			
6	0.75	0.76	0.75	0.79	0.81	1.00																		
7	0.68	0.68	0.73	0.74	0.70	0.79	1.00																	
8	0.72	0.76	0.75	0.82	0.84	0.84	0.81	1.00																
9	0.68	0.74	0.72	0.83	0.80	0.78	0.76	0.84	1.00															
10	0.68	0.69	0.68	0.68	0.71	0.76	0.74	0.77	0.71	1.00														
11	0.67	0.75	0.77	0.77	0.81	0.83	0.75	0.85	0.81	0.78	1.00													
12	0.66	0.63	0.68	0.68	0.69	0.72	0.68	0.69	0.64	0.69	0.68	1.00												
13	0.65	0.61	0.57	0.65	0.63	0.68	0.62	0.66	0.61	0.63	0.61	0.75	1.00											
14	0.69	0.67	0.64	0.69	0.73	0.70	0.74	0.77	0.70	0.66	0.71	0.69	0.67	1.00										
15	0.72	0.71	0.73	0.73	0.76	0.78	0.75	0.76	0.70	0.73	0.75	0.72	0.67	0.79	1.00									
16	0.62	0.64	0.66	0.69	0.73	0.73	0.71	0.71	0.73	0.73	0.77	0.68	0.58	0.69	0.76	1.00								
17	0.65	0.65	0.67	0.71	0.69	0.72	0.73	0.72	0.69	0.66	0.69	0.79	0.73	0.70	0.73	0.64	1.00							
18	0.65	0.67	0.68	0.71	0.74	0.79	0.73	0.76	0.71	0.69	0.76	0.68	0.65	0.72	0.77	0.74	0.71	1.00						
19	0.69	0.71	0.67	0.70	0.69	0.72	0.69	0.78	0.67	0.70	0.72	0.70	0.68	0.68	0.70	0.66	0.71	0.69	1.00					
20	0.61	0.67	0.69	0.71	0.72	0.75	0.73	0.77	0.75	0.66	0.72	0.66	0.59	0.67	0.73	0.73	0.66	0.72	0.67	1.00				
21	0.63	0.66	0.70	0.72	0.67	0.72	0.74	0.73	0.74	0.66	0.69	0.64	0.62	0.68	0.74	0.66	0.64	0.69	0.64	0.75	1.00			
22	0.63	0.68	0.72	0.69	0.69	0.77	0.76	0.74	0.73	0.68	0.73	0.66	0.59	0.67	0.71	0.72	0.67	0.74	0.68	0.75	0.75	1.00		
23	0.63	0.59	0.65	0.68	0.66	0.69	0.68	0.67	0.66	0.61	0.63	0.66	0.60	0.60	0.67	0.65	0.63	0.63	0.59	0.68	0.73	0.71	1.00	
24	0.62	0.62	0.67	0.62	0.61	0.69	0.67	0.64	0.63	0.65	0.60	0.67	0.62	0.63	0.68	0.62	0.65	0.64	0.63	0.65	0.68	0.68	0.65	1.00

Note: Serial number (1 to 24) of the local maize varieties are same as given in Table 1.

high degree of genetic similarity (0.85) among the maize varieties indicated their possible relatedness though they belong to different geographical locations. It might be due to migration by local farmers' or cultivation of varieties in different areas. The dendrogram (Fig. 3) using UPGMA based on the Jaccard similarity co-efficient divided 24 varieties into 3 major clusters (A,B,C) and these major clusters were divided into 8 subgroups. The major cluster A was further divided into 6 subgroups. The subgroup I consists of only one variety Dharap Local-4. The uniqueness of variety was also observed by P-3 and P-10 random primers that they generated unique MIP for this variety. The subgroup II consists of 7 varieties in which Khusalpur Local-4 and Nunawala Local-5 showed most similar varieties (Bootstrap value 29) although they are from different geographical locations. Paschim Khera Local-2 and Khusalpur Local-6 also showed closeness to each other but Kirtinagar Local-4 is non-clustered in subgroup II and also well defined by PCA value (Fig 1). Purvi Khera Local-1 clustered individually in subgroup 3 and showed unique MIPs from P-1, P-3 and P-10 primers also. Bailparow Local-4 and Bhadarabad Local-4, though they are from different geographical locations but clustered together (52%) along with Bagauli Local-1 in subgroup IV. Khusalpur Local-3, Khera Local, Chamoli Local and Quarali Local-1 were clustered together in subgroup V. Nunawala Local-1 and Bhadarabad Local-5 were clustered together in subgroup VI. The major cluster B consists of only one subgroup and having Nunawala Local-6, Bhadarabad Local-8 with similarity 78% and Doiwala Local-2 and Bishanpur Local-2. The reference varieties Pragati and HQPM1 were clustered together

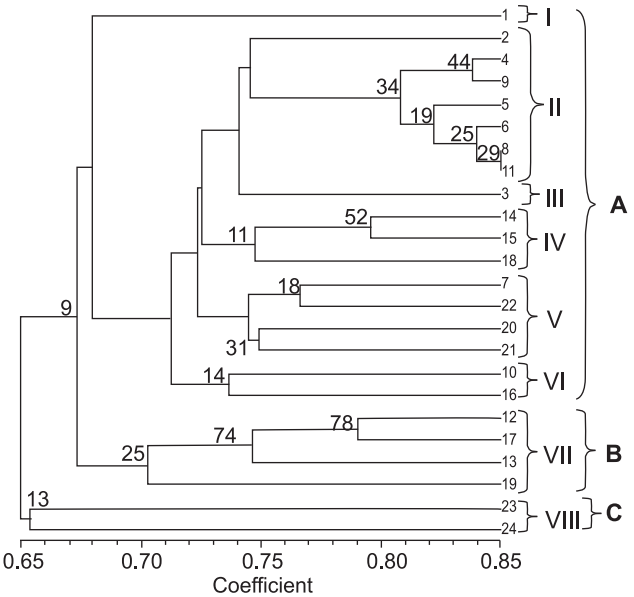

Fig. 3. UPGMA cluster analysis of 24 maize varieties on the basis of RAPD profiles. The values mentioned in the nodes of the diagram are the Bootstrap values. (1-24: Names of varieties as mentioned in Table 1)

but their uniqueness was shown in three dimensional graph of PCA (Fig 1).

Thus, out of a total of 24 varieties unique identification profiles were obtained for 14 varieties while with DUS morphological descriptors only 11 varieties could be discriminated. However, when both morphological and RAPD molecular markers were taken together then 17 varieties could be discriminated. By this study, it can be concluded that in situations where the morpho-physiological DUS descriptors are not able to establish distinctiveness of a variety then molecular markers may be used as additional or complementary descriptors for resolving distinctiveness of maize varieties for granting plant variety protection under PPV&FR Act.

Results of this study provided sufficient evidence that molecular markers would increase the standards of DUS testing if these are included as additional descriptors and the same had been observed in earlier studies on rice (Patra *et al*., 2010; Joshi *et al*., 2010) sorghum (Joshi *et al*., 2011) and maize inbred lines (Gunjaca *et al*., 2008). Higher discrimination power of molecular marker is generated by more balanced distribution of allele frequencies. These results which have been tested and confirmed, shows that molecular markers are ideal additional descriptors for establishing distinctiveness of closely related maize varieties, that serve the purpose of protection and registration of plant variety. The molecular profiling and unique molecular fingerprinting carried out in the present study could be utilized for germplasm conservation, authentication, purification and identification of varieties for registration to protect plant varieties and farmers' rights.

Acknowledgements

The authors are grateful to Department of Science and Technology, New Delhi for providing financial assistance during the period of work.

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