Indian Journal of Agricultural Sciences **86** (12): 1571–6, December 2016/Article
https://doi.org/10.56093/ijas.v86i12.65404

Optimization of ISSR-PCR system and assessing genetic diversity amongst turf grass (*Cynodon dactylon)* mutants

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Received: 18 February 2016; Accepted: 30 May 2016

ABSTRACT

Cynodon dactylon Pers. is highly valued warm season turf grass having global adaptability, robustness and resistance to trampling. In the present study, the ISSR protocol was standardized and quantities of template DNA, dNTPs, $MgCl_2$, Taq DNA polymerase, primer concentration and annealing temperature for each primer were worked out. The reproducible amplifiable products were obtained in all PCR reactions. Analysis of molecular variance (AMOVA), Genetic diversity, Nei's gene diversity, Shannon's index, and Nei's unbiased genetic distance, partition, within- and among-group, of all parameters was analyzed. Levels of genetic divergence between samples were calculated with the fixation index PhiPT. Statistics with AMOVA revealed 1 and 99 % variance among and within various mutants, respectively. Cluster analysis based on the Unweighted-Pair Group Method arithmetic Average (UPGMA), principal coordinate analysis (PCA) and Spatial correlation is a measured that looks at the relationship (genetic distance) amongst mutants. PCOA analysis of ISSR data showed that the first three factors comprised about 75.20% of total variance when the first, second and third axis comprised about 36.64, 23.96 and 14.63% of total variance, respectively. Variation within mutants was the maximum in DFR-C-448 followed by DFR-C-446(10.357). In DFR-C-448 unique number of bands to a single population was observed. Correlogram plot shows that there is significant positive genetic structure at distance class of mutants DFR-C-448.

Key words: *Cynodon dactylon,* Genetic diversity, IISR-PCR, ISSR markers

Turf grasses are used worldwide for lawns at home, offices, athletic fields and playgrounds, recreational facilities and roadsides (Tiwari *et al*. 2015a). In India Bermuda grass (*Cynodon dactylon* Pers.) is widely used as turf grass, and is easy to grow, hardy and effective in soil conservation (Tiwari *et al.* 2014a). There is an increasing demand for new and improved cultivars to meet environmental restrictions and challenges (Tiwari *et al.* 2015b). This results in the development and release of many turf grass cultivars each year. Reliable cultivar identification becomes paramount to distinguish cultivars, maintain varietal purity and to protect plant breeder's rights (Tiwari *et al*. 2015b).

DNA based markers are available for understanding genetic variations in plant genomes. Thus, DNA finger printing for varietal identification has become an important tool for establishing genetic signature in plant breeding and germplasm management (Singh *et al*. 2014). Markers such as inter-simple sequence repeats (ISSR) have been widely used in genetic diversity studies because these need no prior DNA sequence information, development cost is low, and laboratory procedures can easily be transferred across plant species (Barth *et al.* 2002). This technology has been used to DNA fingerprint a wide range of crops (Farsani *et al.* 2012**)** and to understand ploidy complex and the geographic origin of many plant species (Gulsen *et al.* 2009, Arcade *et al*. 2004, Farsani *et al.* 2012). DNA profiling techniques that have been successfully used in assessing relatedness of *Cynodon* accessions includes DNA amplification fingerprinting (DAF) (Anderson *et al.* 2001), RAPD (Etemadi *et al.* 2006, Roodt *et al.* 2002), AFLP (Karaca *et al.* 2002), ISSR (Wu *et al.* 2004, Farsani *et al.* 2012) and SSR (Kamps *et al.* 2011). In this study, we optimised protocol and analyzed genetic diversity of Bermuda grass using ISSR markers, aiming to assess the genetic diversity and obtain more DNA markers with higher resolution for genetic studies and management of the species.

MATERIALS AND METHODS

Thirty uniform stolen (sprigs) sets of propagules of *Cynodon dactylon* (local turf grass) were irradiated with nine doses of gamma rays from CO_{60} source for each treatment at National Physical Laboratory (NPL), Indian Agriculture Research Institute (IARI), New Delhi. Each treated sprig was planted in a pot and further clonally multiplied at Research Field of Directorate of Floriculture Research, IARI campus, New Delhi. Five promising variants, i.e. *DFR-NS-I, DFR-C-440, DFR-C-444, DFR-C-446* and

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Table 1 Optimization of the ISSR-PCR reaction parameters for five mutants of *Cynodon dactylon*

PCR parameter	Tested range	Optimum conditions
DNA template concentration (ng)	10, 20, 30, 40, 50, 75, 100, 150, 175, 200	75 ng
Magnesium chloride (mM)	1.0, 1.5, 2.0, 2.5, 3.0	2.5 mM
Deoxynucleotide triphosphates (dNTPs) (mM)	0.2, 0.4, 0.6, 0.8	0.6 mM
Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5	0.5 µM
Taq DNA polymerase (units)	0.5, 1.0, 1.5, 1.75	1 U
Annealing temperature (°C)	55 and 56	55 or 56°C for different primer

DFR-C-448 were selected. For ISSR analysis samples were collected and experiment were laid out during 2013-14 in the Molecular Laboratory of Plant Pathology division of IARI New Delhi. Fresh young leaves of 1.5 g were harvested for DNA isolation.

Fresh young leaves weighing 1.5 g were harvested and wrapped in foil paper for DNA isolation. These were immediately taken to the laboratory and kept in refrigerator at -20°C to retain freshness of the material. The leaves were vigorously rinsed in distilled water to remove particles on leaf surfaces. About 200 mg of each sample was gently ground into paste in a mortar with 2 ml cTAB extraction buffer (pre-heated at 65°C for 10 min). To facilitate and speed up grinding, leaves were chopped into smaller bits with scissors. As a precaution, scissors were dipped in absolute ethanol before reuse. After grinding, equal volume (approximately 1 ml each) of the resultant paste was distributed into two separate 2 ml microcentrifuge tubes. This stage took 3 to 5 min/sample.

A volume of 2 µl β-mercaptoethanol was added to each tube, which was then mixed thoroughly by gently rocking the rack. Samples were incubated in a water bath at 65°C for 30 min and allowed to return to room temperature for 5 to 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) (i.e., 1 ml into each tube) was added for extraction. This was mixed gently by continuously shaking and inverting the tubes for up to 5 min. Samples were centrifuged at 11,000 rpm for 10 min at 25°C to separate the phases. The top light green-colored aqueous phase was transferred to new 1ml microcentrifuge tubes, along with 0.75 volume chilled isopropanol (e.g. for 1 ml aqueous phase, 750 µl chilled isopropanol added) to precipitate the DNA. Samples were mixed gently by continuous inversion, kept at -20°C overnight followed by centrifugation to recover the DNA pellets.

The samples were centrifuged at 12 000 rpm for 10 min at 4°C. Carefully, the supernatant was discarded being mindful of the DNA pellet; the pellets were washed in 70% ethanol and air-dried until ethanol evaporated completely from the samples. This was facilitated by inverting tubes on tissue paper or paper towel or using a vacuum blower. The DNA pellets were rehydrated/dissolved in 400 µl T10E1 buffer and treated with 3 µl RNase (10 mg/ml). Samples were incubated for 50 min at 37°C. For purification, 400 µlphenol:chloroform:isoamyl alcohol (25:24:1) was added followed by centrifugation for 5 min at 10 000 rpm. The top layer was carefully pipetted into new 1.5-ml microcentrifuge tubes, with the addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifugation at 5 000 rpm for 5 min at 22°C. The supernatant was transferred to new 1.5-mL tubes with the addition of 0.6 volumes (0.6 × 300=180 µl) of chilled is opropanol followed by several but slow inversions of the tubes. Sodium acetate (3 M) 1/10 volume (30 µl), was added to facilitate DNA precipitation. DNA was further precipitated at -20°C for overnight. A volume of 200 µl 70% ethanol was added onto the pellet with centrifugation at 12 000 rpm for 10 min at 4°C. The supernatant was discarded, and the pellet dried and dissolved in TE (volume of TE depended on the visible quantity of pellet in the tube).

DNA yield and purity were determined by agarose gel electrophoresis and spectrophotometric analyses methods. Aliquots (1 µl) of DNA samples were run on a 0.8% agarose gel and compared with band intensities from known concentration of lambda DNA standards. The yield was further measured by checking the optical density (OD) in a UV spectrophotometer at 260 nm. DNA purity was determined by calculating the absorbance ratio at A260/280.

Based on the previous study of Hu *et al*. 2011, 28 ISSR primers (Table 2) were used for analysis. The reactions were carried out in a DNA Thermocycler PTC-200. Five factors including Taq DNA polymerase, dNTP, primer concentration, Mg^{2+} and annealing temperatures were investigated as described in Table 1. Reactions without DNA were used as negative controls. Each 25 µl reaction volume contained 10 mMTris-HCl (pH 8.3), 50 mMKCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.6 mM dNTPs, 0.5µM primer, 1 unit Taq Plus DNA polymerase and approximately 75 ng template DNA (Table 1). Gels with amplification fragments were visualized and photographed in UV light by using Gel Documentation System. DL 2000 ladder was used as reference DNA molecular weight. Optimized conditions for ISSR-PCR protocol are given in Table 1. The optimized annealing temperatures for the 28 primers were validated by using thermocycler T-gradient.

ISSR amplified fragments, with the same mobility according to molecular weight (bp), were scored manually for band presence (1) or absence (0). Data recording followed the three principles: 1) Only the easily recognizable bands can be recorded, and the obscure bands are excluded; 2) the bands that cannot be precisely identified should be excluded; and 3) the bands with the same mobility but different intensity should not be treated as the same bands (Weising *et al*. 2005). Analysis of molecular variance (AMOVA), Genetic

Table 2 Characteristics of the 28 ISSR primers used for the detection of polymorphism in five mutants of turf grass

Primer	Sequence (5' → 3')	Annealing temperature (°C) *TA* (°C)	No. of polymorphicloci scored/ No. of loci scored	% of poly-morphism bands	Size range of fragments (bp)
UBC807	(AG)8T	56	7/10	70	350-1700
UBC817	(CA)8 A	56	5/9	55.55	300-1900
UBC821	(GT)8 T	56	6/8	75	400-1000
UBC823	(TC)8C	56	6/10	60	100-1800
UBC835	(AG)8GCC	56	6/9	66.66	350-1700
UBC836	(AG)8CTA	56	6/9	66.66	450-1700
UBC840	(GA)8CTT	56	6/11	54.54	270-1800
UBC842	(GA)8CTG	56	6/8	75.00	260-1800
UBC849	(GT)8CTA	56	5/10	50.00	250-1600
UBC855	(AC)8 CTT	56	6/8	75.00	400-2000
UBC856	(AC)8 CTA	56	8/10	80.00	300-1400
UBC857	(AC)8CTG	56	6/10	60.00	370-1500
UBC873	(GACA)4	56	4/9	44.44	600-1800
UBC880	(GAGA)3	56	7/10	70.00	300-2000
P 1	(GA)8CTA	55	9/9	100	450-1500
P 2	(GA)8AGC	55	10/10	100	600-2000
P 3	(GGGGT)3	55	8/8	100	250-1900
P 4	(AC)8GCT	55	6/6	100	500-1000
P 5	(AC)8TG	55	9/9	100	270-1800
P 6	(TCC)5TG	55	8/8	100	400-2000
P 7	(AC)8GT	55	10/10	100	270-1300
P 8	(AG)8TC	55	8/10	80	400-1600
P 9	(GA)8GCC	55	9/9	100	300-1600
P 10	ACT ACG ACT (TG)7	55	10/10	100	500-2000
P 11	ACTCGTACT (AG)7	55	6/10	60	400-1700
P 12	CGT AGT CGT (CA)7	55	8/10	80	500-1000
P 13	AGT CGT AGT (AC)7	55	8/10	80	400-1500
P 14	(AC)8CG	55	9/9	100	350-750

diversity within and among populations was measured as the percentage of polymorphic bands, Nei's gene diversity (Nei 1973), Shannon's index and Nei's unbiased genetic distance, partition.

With in- and among-group, of all parameters was analyzed using the version 6.5 of software in GenALEx (Peakall and Smouse 2006). Phi-statistics (PhiPT) is a modified version of Wright's F that refers to the relative contributions of between-population separation to the overall genetic variation in the whole sample. Levels of genetic divergence between samples were calculated with the fixation index PhiPT (Excoffier *et al.* 1992), an estimator that includes information on haplotype frequency and molecular distance (calculated with GenAlex, ver. 6.5). Cluster analysis based on the Unweighted-Pair Group Method arithmetic Average (UPGMA), principal coordinate analysis (PCA) and spatial correlation is a measured that looks at the relationship (genetic distance) amongst mutants and correlogram prepared to show the results of the fine-scale spatial auto correlation analysis where genetic correlation values (*r*) are plotted as a function of distance. U and *L* represent 95% CIs around the null hypotheses of no structure (GenAlex, ver. 6.5).

RESULTS AND DISCUSSION

ISSR Amplification

The 28 selected ISSR primers produced 257 bands with an average of 9.17 bands per primer, of which 201 (78.21%) were polymorphic ranging from 100 to 2 000 bp in size (Table 2). Each primer produced 4 to 11 polymorphic bands with an average of 6.28.The most polymorphism was shown by nine primers (P1, P2, P3, P4, P5, P6 P7, P9 and P10), which showed 100% polymorphism. Statistics with AMOVA revealed 1 and 99 % variance among and within various mutants, respectively. Variance differentiation was significant ($P < 0.01$) for all variants (Table 3). Variation within mutants was the maximum in DFR-C-448(25.00) followed by DFR-C-446(10.357). This result suggests that genetic variance was high within mutants and low between various mutants.Phi-statistics values ranges from 0.014 to 0.918 which refers to the relative contributions of between-population to the overall genetic variation in the whole sample of *Cynodon dactylon*. The greater the Fst values, greater the differences between mutants. The monomorphic bands of low degree of similarity indicated high divergence between the Bermuda

Table 3 Analysis of molecular variance (AMOVA) of profiles developed from inter-simple sequence repeats markers in five mutants of *Cynodon dactylon* turf grass

Source of variation	df	Sum of squares	Variance of component(MS)	Est. Var.	Percentage of variation	*P*-value[a]
Among Pops	4	2.300	0.575	0.006	1%	<0.01
Within Pops	135	55.286	0.410	0.410	99%	<0.01
Total	139	57.586		0.415	100%	
Phi-statistics	*Value*		*P(r and >= data)*			
PhiPT	0.014		0.133			
PhiPT max	0.918					
Phi'PT	0.015					

Variation within population	*DFR-NS-1*	*DFR-C-440*	*DFR-C-444*	*DFR-C-446*	*DFR-C-448*	*Total SS within population*
SSWP	6.643	6.643	6.643	10.357	25.000	55.286

[a]Levels of significance were obtained through nonparametric procedures using 99 permutations. PhiPT = AP / (WP + AP) = AP / TOT. Where, AP = Est. Var. Among Pops, WP = Est. Var. Within Pops. Probability, P(r and >= data), for PhiPT is based on standard permutation across the full data set.

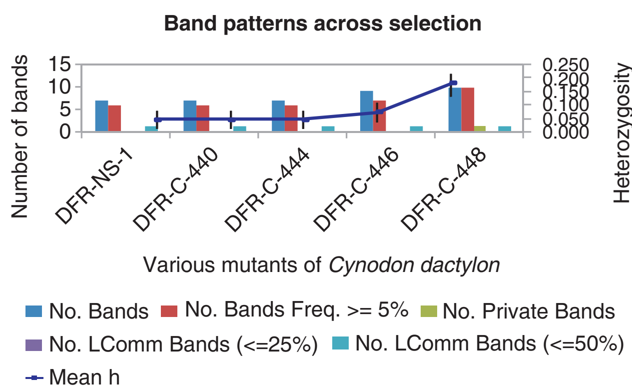

Fig 1 DNA Band patterns across selection of *Cynodon dactylon*

mutants. In DFR-C-448 unique number of bands to a Single Population was observed (Fig 1).

Genetic variation and similarity among genotypes

The average values of observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (He) and Shannon's information index (I) for all primers were 2, 1.108, 0.079 and 0.135, respectively (Table 4). Scatter plot matrices are a great way to roughly determine the linear correlation between multiple variables. This is particularly helpful in pinpointing specific variables that might have similar correlations to genomic or proteomic data. In Fig 2 of scatter plot matrix of variables have been written in a diagonal line from top left to bottom right. Then each variable is plotted against each other. In this scatter plot, there is a correlation amongst mutants of *Cynodon dactylon* because the plot looks like a line. There is probably less of a correlation between DFR-NS-1 and other variants. More statistical analyses would be needed to confirm or deny this. The maximum correlation exists amongst DFR-C-440, DFR-C-446 and DFR-C-446(r=0.9554, 0.9330 and 0.5097) and it was least with DFR-NS-1.

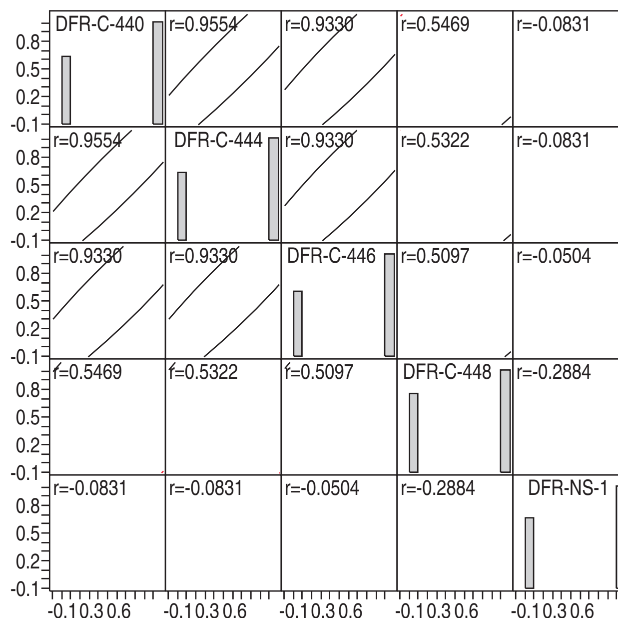

Fig 2 Scatterplot matrix of multivariate correlations

Cluster analysis

The pair-wise genetic distance estimates of the turfgrass cultivars in this study were analyzed and are given in Table 5. PCOA analysis of ISSR data showed that the first three factors comprised about 75.20% of total variance when the first, second and third axis comprised about 36.64, 23.96 and 14.63 % of total variance respectively. Ward dendrogram clustering of ISSR data produced similar results supported by PCOA ordination plot. The highest value (r = 0.9554) was observed for the unweighted paired group method with arithmetic average (UPGMA) clustering method.

Spatial correlation is a measured that looks at the relationship (genetic distance) amongst mutants. Correlogram (Fig 3) showed the results of the fine-scale

Table 4 Variation of genetic parameters developed from inter-simple sequence repeats markers for different mutants of *Cynodon dactylon*

Population	DFR-NS-1	DFR-C-440	DFR-C-444	DFR-C-446	DFR-C-448	Grand
No. of alleles Na						
Mean	2.000	2.000	2.000	2. 00	1. 00	2.0
Standard deviation	0.258	0.258	0.258	0.221	0.153	0.108
Effective number of alleles, *Ne*						
Mean	1.066	1.066	1.066	1.096	1.246	1.108
Standard deviation	0.050	0.050	0.050	0.050	0.063	0.025
Nei's gene diversity, *He*						
Mean	0.047	0.047	0.047	0.074	0.179	0.079
Standard deviation	0.033	0.033	0.033	0.034	0.043	0.017
Shannon's information index, *I*						
Mean	0.083	0.083	0.083	0.134	0.294	0.135
Standard deviation	0.053	0.053	0.053	0.055	0.068	0.027

Table 5 Pearson correlation coefficients *Cynodon dactylon* mutants

Correlations	DFR-C-440	DFR-C-444	DFR-C-446	DFR-C-448	DFR-NS-1
DFR-C-440	1.0000	0.9554	0.9330	0.5469	-0.0831
DFR-C-444	0.9554	1.0000	0.9330	0.5322	-0.0831
DFR-C-446	0.9330	0.9330	1.0000	0.5097	-0.0504
DFR-C-448	0.5469	0.5322	0.5097	1.0000	-0.2884
DFR-NS-1	-0.0831	-0.0831	-0.0504	-0.2884	1.0000

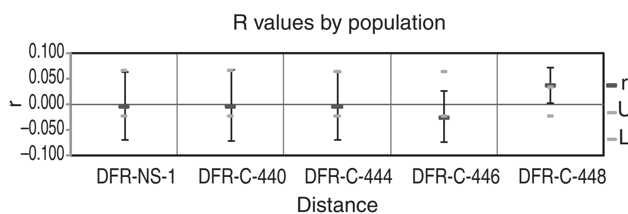

Fig 3 Correlogram showing the results of the fine-scale spatial autocorrelation analysis where genetic correlation values (r) are plotted as a function of distance

spatial autocorrelation analysis where genetic correlation values (*r*) are plotted as a function of distance. *U* and *L* represent 95% CIs around the null hypotheses of no structure. The plot shows that there is no significant positive genetic structure at any distance class of mutants except DFR-C-448.

The present optimized protocol for DNA isolation and ISSR-PCR technique may serve as an efficient tool for further molecular studies in bermuda turf grass. The modified cTAB was the better choice especially for a large DNA samples which was necessary for the study on genetic diversity, but it often need to be slightly modified even if it had been reported in other laboratory. This may be attributed to compounds difference of leaf tissue in different regions, chemicals and solutions manufactured by different company. After obtaining a total genomic DNA with high purity and yield, primers still need to be screened for conformity because the same primer may exhibit different amplification results in different species. Therefore, primers screening and optimal PCR system based on each selected primer are necessary. In the present study, DNA isolated by modified cTAB method yielded strong and reliable amplification products showing its compatibility for the 28 selected primers of ISSR-PCR. The present optimized protocol for ISSR technique may serve as an efficient tool for further molecular studies. Induction and evaluation of diversity among and within populations would be of great significance for their utilization in breeding program and *in situ* conservation. In recent years, genetic markers are increasingly used for the study of genetic diversity. Moreover, the polymorphism determined by these markers is one of the valuable parameters for study of populations and understanding of their genetic differences. The high reproducibility of ISSR markers may be because of using longer primers and higher annealing temperature. Based on its unique characters, ISSR technique can detect more genetic loci than isozyme and has higher stability than others (Kameli *et. al*. 2013).

The polymorphic information content (PIC) and the polymorphism rate (*P*) were used to measure the genetic diversity in bermuda grass accessions. High, medium or low polymorphism is in accordance with PIC > 0.5, 0.5 > PIC > 0.25 and PIC < 0.25, respectively (Farsani *et al*. 2012). Moreover, the mean value of the PIC obtained in this study was 62.85, indicating that the primers could develop polymorphism which is useful for genetic variation of genotypes studied in this research. The average values of Na, Ne, He and I indicated that the background genetic data of germplasm accessions should be considered for integrated application in the breeding programs for germplasm improvement. It should be noted that, as a measure of genetic variation, the effective number of alleles (Ne) or expected heterozygosity (He) is more appropriate than the actual number of alleles (Na), since the latter depend on the sample size (Tajjima *et al*. 1994). Artificial mutation is one of the most common methods used to create variation in bermuda grass (Tiwari *et al*. 2015). In this study, we included four mutants, which were divided into two subgroups on the UPGMA tree. Among these mutants, DFR-C-448 and DFR-

NS-1 formed separate groups that are consistent with the results of this study. This constructed dendrogram may be able to discriminate between genetically different chromosome numbers of *Cynodon dactylon* as reported by Anderson *et al*. (2001), Etemadi *et al*. (2006) and Gulsen *et al*. (2009).

In summary, our results indicate that the level of polymorphism among bermuda grass mutants is appreciably high. The results of this study also suggest that the ISSR marker offers a powerful means to analyze the genetic diversity among accessions. Cluster analysis based on the Unweighted-Pair Group Method arithmetic Average (UPGMA), principal coordinate analysis (PCA) and Spatial correlation are measures that look at the relationship (genetic distance) amongst mutants. PCOA analysis of ISSR data showed that the first three factors comprised about 75.20% of total variance when the first, second and third axis comprised about 36.64, 23.96 and 14.63 % of total variance, respectively. Variation within mutants was the maximum in DFR-C-448 followed by DFR-C-446(10.357). In DFR-C-448 unique number of bands to a Single Population was observed. Correlogram plot shows that there is significant positive genetic structure at distance class of mutants DFR-C-448.

ACKNOWLEDGEMENT

We are grateful to the assistance provided by Dr S C Dubey, Principal Scientist, Plant Pathology, ICAR–IARI, New Delhi for this study.

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