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Allelic Diversity Among Short Duration Maize (*Zea mays* L.) Genotypes Using SSR Markers

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In the present investigation, 25 lines of maize were analysed for allelic diversity using 15 carotenoids specific simple sequence repeats (SSRs) markers including gene targeted marker y1SSR. All the markers were found to be polymorphic thus able to differentiate different genotypes. Total of 66 alleles were detected across the 15 polymorphic loci with an average of 4.4 alleles per locus. Polymorphic information content (PIC), a measure of diversity analysis, ranged from 0.63 (umc 2047) to 0.83 (y1SSR). Jaccard's similarity coefficient was found to vary from 0.16 (between CM-300 and LPP-1) to 0.65 (between Amar and Pragati). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram generated using Jaccard's similarity coefficients of SSR markers data divided 25 genotypes into two broad groups. Both the groups were further sub-divided into seven clusters. Molecular marker based diversity did not correspond to quantitative estimates of kernel carotenoids.

Key words: Maize, carotenoids, SSR markers.

Genetic diversity evaluation is frequently used by the breeders as an alternative germplasm selection method for identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection, and to introgress desirable alleles from diverse germplasm into the available genetic base. Among the various PCR based markers available for germplasm characterization, the microsatellites or simple sequence repeats (SSRs) markers are widely preferred in maize for diversity analysis (Prasanna and Hoisington 2003). Due to their high allelic diversity and genetically co-dominant nature, the SSR loci are also well-suited for the study of population structure (Garris *et al.* 2005). Allelic polymorphisms are important for mining desirable allele and further its marker assisted incorporation in otherwise better genetic background for enhancing genetic value in general, and biofortification in particular in maize. Maize exhibits considerable natural variation for kernel carotenoids, with some lines accumulating as much as 66 mg/g of carotenoids (Harjes *et al.* 2008). However, this necessitates that allelic variants in maize germplasm must be characterized and quantified so that the desirable alleles could be made available in breeding programme for biofortification (Menkir *et al.* 2008). Yellow kernel maize is the only among major cereals which have carotenoids in its kernel however kernel colour in maize (different shades of yellow) is not associated with total carotenoids or provitamin A carotenoids (Harjes *et al.* 2008). The kernel colour, therefore, can not be used reliably in selection of high kernel carotenoids lines. The genomics approaches have identified many SSR markers linked to the QTLs spread across the genome and responsible for total carotenoids as well as different forms of carotenoids (Chander *et al.* 2008). Such SSR markers have been developed using mostly temperate germplasm, however, they may be useful in understanding the diversity in short duration subtropical maize with varying carotenoids. With this intention, the present investigation was undertaken to analyze a set of 25 maize genotypes with 15 SSRs/microsatellites markers to study the molecular genetic diversity and analyse its correlation with quantitative data in short duration subtropical maize.

Materials and Methods

Seed materials

The materials for the present investigation comprised of 25 maize lines (18 inbred lines and 7 composite varieties). All the lines were of early maturity group. The inbred lines YHPA-1, YHPA-2 and YHPA-3 were derived from yellow heterotic pool A (YHP-A) whereas inbred lines YHPP-1, YHPP-4 and YHPP-9 have been derived from yellow heterotic pool B (YHP-B). The inbred lines Pop31-1, Pop31-5, Pop31-6 and Pop31-7 were derived from Population 31. The inbred lines Pob446-2 and Pob446-3 were derived from Population 446. The CM (Coordinated Maize) lines used in the study were developed under the Coordinated Maize Improvement Programme and designated by

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Directorate of Maize Research, New Delhi. The other materials used namely Amar, D-131, Surya, D-765, Pragati, Kanchan, Tarun are varieties developed and released by Pantnagar. The inbred line CM-300 had white grain colour whereas the remaining of the lines had yellow (different shades) to orange kernel colour. The kernel sizes were small to bold with round to tooth shaped flint/semi-flint kernel. Seeds of all the 25 genotypes were taken from maize improvement programme of G. B. Pant University of Agriculture and Technology, Pantnagar.

Extraction of total carotenoids

Fifty grams (g) of well dried seeds of each genotype were ground to fine powder using grinder and stored in butter paper bag at 4^0C. 0.5 g of fine powder was taken in Falcon tube and 6 ml of Ethyl Alcohol:2, 6-Di-tert-butyl-4-methylphenol (EtOH:BHT) was added to each sample and mixed by vortexing. Samples were incubated at 85^0C in water bath for 5 minutes and were mixed again after 3 minutes by vortexing. Freshly prepared 120 µl KOH (1g KOH/ml H_2O) was added to each sample. Saponification was done by incubating samples for 5 minutes at 85^0C in water bath, vortexed for 10 seconds and further incubated at 85^0C for 5 minutes. Samples were cooled down on ice and 4 ml of H_2O was added. Further 3 ml of Petroleum Ether (PE):Diethyl Ether (DE) (2:1, v/v) was added to each samples. Samples were mixed by vortexing and thereafter centrifuged at 1400g for 10 minutes. Upper phase (supernatant) of the mixture was pipette out and transferred to fresh Falcon tube. Mixing of 3 ml of PD:PE (2:1, v/v) and centrifugation steps were repeated thrice. Finally, approximately 8 ml of extract was recovered from each sample. One ml aliquots from each sample were taken in quartz cuvette and their optical density (OD) at 450nm against petroleum ether: diethyl ether (2:1, v/v) as blank, was recorded using UV-Visual Dual Beam Spectrophotometer. The OD value of each sample was used to determine the carotenoids concentration using Lambert-Beer equation as described below:

$E = \sum c\, d$

Where,

E = extinction (Spectrophotmeter reading)

ε = molar extinction

coefficient c = concentration

d = distance =1, to determine concentration

$c = E/\varepsilon$

The total kernel carotenoid was extracted following the protocol of Schaub *et al*. (2004).

DNA extraction

One gram fine powder of maize kernel of each genotype was further ground in mortar-pestle using liquid nitrogen.Total genomic DNA was extracted using standard CTAB method (Doyle and Doyle 1987) with slight modification such as the use of 5.0 M Potassium Acetate in extraction buffer. The isolated DNA was quantified using double beam UV-Vis spectrophotometer. The absorbance was measured at 260 nm and the OD value was used to calculate the quantity of DNA.

PCR amplification

The PCR reaction mixture for each sample was prepared in 25 µl volume which consisted of 2.5 µl Taq buffer (10X), 1.0 µl dNTPs mix (10mM mix), 1.5 µl $MgCl_2$ (25 mM), 1.0 µl Taq polymerase (1.0 unit/ µl),1.2 µl forward primer, 1.2 µl reverse primer, 2.0 µl template DNA and 14.6 µl double distilled water. The PCR amplification was performed in an Eppendorf Thermalcycler where initially DNA was denatured at 94ºC for 5 min. Further, 35 cycles of denaturation at 94ºC for 1 min., annealing of the primer at XºC (where XºC varied from 56ºC to 59ºC) for 1:30 min. and an extension period for 2 min. at 72ºC were performed to amplify the SSR loci. The final extension was allowed at 72ºC for 5 min after completion of 35 cycles. The carotenoids specific SSR primers used in the investigation were phi091, umc1403, umc2047, umc2115, umc1447, umc1692, bnlg1792, umc2332, umc2313, umc1595, umc2373, umc1553, umc1506, Y1SSR and umc1070 (Chander *et al.* 2008).

Electrophoresis

Initially PCR products were separated on agaraose gel, however, the small allelic variation could not be resolved. The polyacrylamide gel electrophoresis (PAGE) was then adopted for better resolution of SSR allelic variations. The denaturing gels composed of 8% of 19:1 acrylamide with 42% urea were prepared and used in Bio-Rad PROTEAN II® system. Prior to sample loading, prerun of gels were performed at 400 V for 20 min. The 10 µl of PCR product was mixed with DNA sequencing stop solution, denatured at 95ºC for 5min. and electrophoresed at 100 V for 4.00 h in 1X-TBE buffer (pH 8.0). The gels were stained with 0.2% silver nitrate solution and documented using Gel Documentation system (CIMMYT 2005).

The data matrix was generated taking presence of band as ‘1’ and absence of band as ‘0’ which was subsequently used for computation of Jaccard’s similarity coefficients, UPGMA clustering algorithm and polymorphic information content (PIC) values using NTSYS-pc 2.02 (Rohlf 1998). Boot strapping was analysed using WINBOOT software. Linear regression was analysed to establish correlation between SSR based groupings and carotenoid content.

Results and Discussion

Carotenoids have been shown to possess complex heredity and hence are difficult to

manipulate genetically using normal breeding methods. The QTL accounting for significant variance for carotenoids along with the candidate gene targeted markers (GTM) from carotenoid biosynthetic pathway may be of great help in marker assisted selection aimed for breeding maize with higher carotenoids level (Chander *et al.* 2008).

The 15 SSR markers specific to QTLs identified to influence kernel carotenoids in maize were used to quantify the molecular diversity among 25 lines of maize. All the SSR markers were found to be polymorphic thus were able to differentiate different genotypes. The marker y1SSR is a phytoene synthase (*psy1*) gene targeted marker tightly linked with major QTL associated with individual as well as total carotenoids in maize (Chander *et al.* 2008). The *y1* mutant is a white endosperm mutant with greatly reduced levels of kernel carotenoids and has been cloned and maps to bin 6.02 in maize.

The data matrix was prepared using a total of 66 polymorphic fragments from 15 primer pairs, equivalent to 4.4 loci/primer (Table 1). The number of alleles detected per locus was relatively higher in the case of y1SSR, umc1553 and bnlg 1792 (each with 6 alleles per locus). The applicability of genetic markers to analyse a trait depends on the extent of polymorphism detectable with the given markers. It is thus necessary to establish the polymorphic information content (PIC), which expresses the ability of a locus to discriminate between the lines. The PIC values of SSR markers used in the study were high, ranging from 0.63 (umc 2047) to 0.83 (y1SSR) with mean value of 0.72 (Table 1). This indicated that SSR markers could be efficiently

Table 1. Number of alleles per locus and PIC value for 15 polymorphic loci

SSR Primer	Chromosome location	Repeat	No. of alleles per locus	PIC value
umc 2313	6.01	$(AG)_6$	5	0.66
umc 1692	5.03	$(CGAT)_4$	4	0.74
umc 1506	10.05	$(AACA)_4$	5	0.79
y1SSR	6.01	CCA; CCATC, TCATC	6	0.83
phi 091	7.03	GCTT-TA	4	0.71
umc 1595	6.02	$(CT)_6$	4	0.75
umc 2332	7.04	$(CTC)_5$	5	0.73
umc 1070	1.02	$(TC)_7$	5	0.71
umc 2373	5.04	$(GCT)_4$	4	0.67
umc 1553	1.11	$(AG)_8$	6	0.77
umc 1403	1.03	$(GCA)_4$	3	0.67
umc 1447	5.03	$(CTT)_4$	3	0.65
umc 2047	1.09	$(GACT)_4$	3	0.63
umc 2115	5.02	$(TGCCA)_5$	3	0.65
bnlg 1792	7.02	$(AG)_{16}$	6	0.79

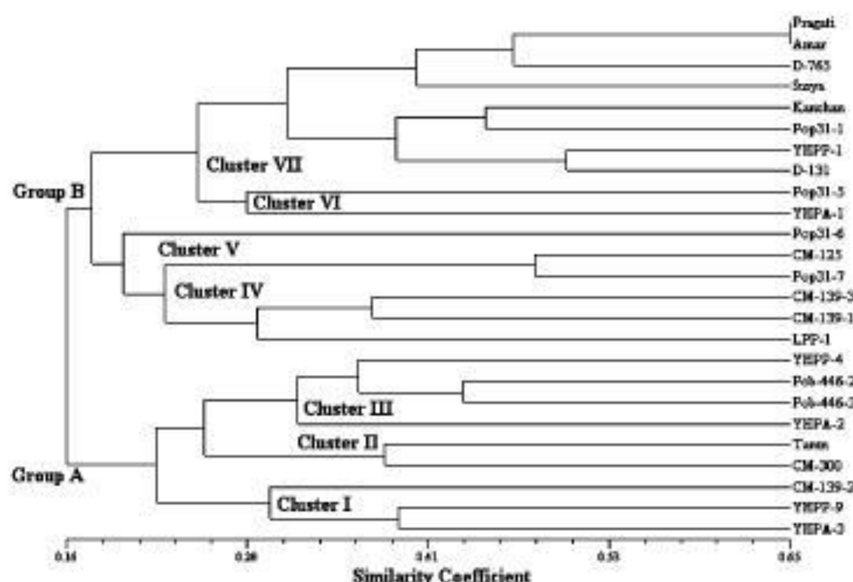

Fig 1. Dendrogram depicting carotenoids allele specific SSR diversity among 25 genotypes of maize.

Table 2. Cluster mean for total carotenoids in maize

Cluster	Genotypes	Carotenoids content (µg/g)	Cluster mean (µg/g)
Group A			
Cluster I	YHPA-3	25.69	22.03
	YHPP-9	19-40	
	CM-139-2	21.01	
Cluster II	Tarun	8.72	04.37
	CM-300	0.027	
Cluster III	YHPA-2	23.47	20.21
	Pob-446-3	19.07	
	Pob-446-2	19.53	
	YHPP-4	18.78	
Group B			
Cluster IV	LPP-1	23.70	23.44
	CM-139-1	17.84	
	CM-139-3	24.33	
	Pop-31-7	25.59	
	CM-125	25.75	
Cluster V	Pop-31-6	23.96	23.96
Cluster VI	YHPA-1	22.59	22.66
	Pop-31-5	22.72	
Cluster VI	D-131	19.28	14.37
	YHPP-1	14.78	
	Pop31-1	9.23	
	Kanchan	8.9	
	Surya	17.73	
	D-765	13.04	
	Amar	21.38	
	Pragati	10.64	

applied to detect polymorphism even with a relatively low number of primers. The markers umc 1692, umc 1506, y1SSR, umc 1595, umc 2332, umc 1553 and bnlg 1792 had PIC values more than the average, while umc 2313, phi 091, umc 1070, umc 2373, umc 1403, umc 1447, umc 2047 and umc 2115 had PIC values less than the average. The high PIC values for SSRs confirm the data already published, indicating that this set of SSR markers are extremely efficient for polymorphism analysis (Smith *et al.* 1997).

Based on the SSR markers data, the pair-wise Jaccard's similarity coefficients were estimated. The similarity coefficients were found to vary from 0.16 to 0.65. The highest value of genetic similarity (0.65) was found between Amar and Pragati whereas the most divergent lines detected were CM-300 and LPP-1. The UPGMA dendrogram was constructed using Jaccard's similarity coefficients of SSR markers data generated on 25 genotypes (Fig. 1). The dendrogram divided 25 genotypes into two broad groups namely Group A (lower) and Group B (upper). These two groups were related to each other by similarity coefficient of ca. 0.16. Cluster I consisted of three genotypes with mean carotenoids of 22.03 µg/g (Table 2). Cluster II had two least carotenoids containing genotypes. Four genotypes of cluster III varied in carotenoids content from 18.78 to 23.47 µg/g. The five genotypes of cluster IV had average carotenoids content of 23.44 µg/g whereas cluster V had one genotype with 23.96 µg/g of carotenoids content. Cluster VI had two genotypes with average carotenoids of 22.66 µg/g. The eight genotypes of cluster VII varied in carotenoids content from 8.9 to 21.38µg/g with average of 14.37 µg/g. The 25 genotypes were therefore classified into 7 clusters based on markers data which indicated the power of SSR markers in detection of allele specific diversity. However, it is also obvious from the clustering pattern that some of the clusters contained lines with low, medium and high carotenoids together.

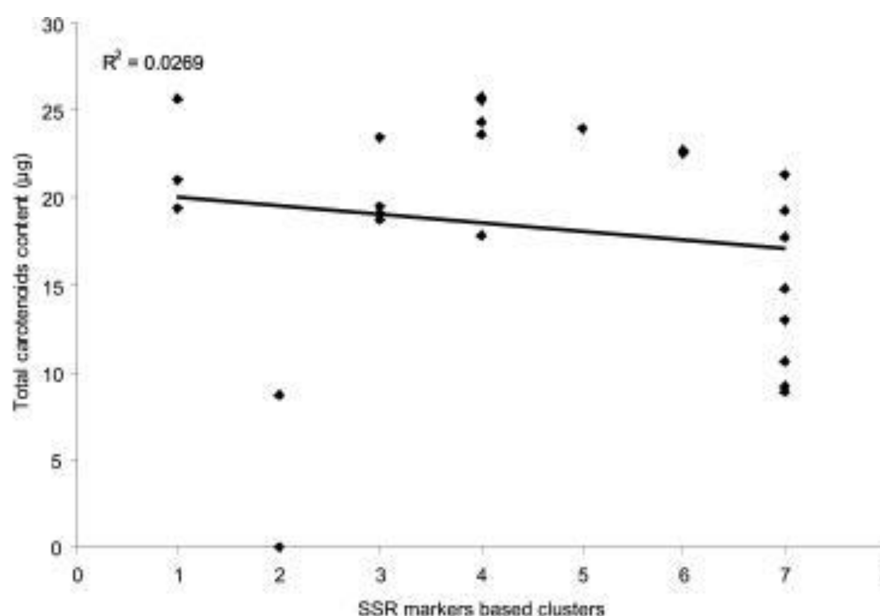

Fig 2. Relation between SSR markers based grouping and kernel carotenoids content in maize

The carotenoids content of each genotype was plotted against the cluster it occupied and linear line was drawn to establish relation of SSR data based clustering with quantitative value of carotenoids (Fig. 2). The linear regression analysis revealed non-significant correlation between SSR based clustering and quantitative data. Further, low coefficient of determination (R^2=0.027) indicated that SSR based clustering pattern had little influence on quantitative estimates of carotenoids. Thus, the data revealed that allelic variations did not differentiate clearly the lines with high, medium and low carotenoids content. One reason of such disparity may be due to different genetic background of the genotypes used in the present investigation. Collins *et al.* (2008) also stated that QTLs from a particular genetic background usually show smaller effects or disappear altogether in different backgrounds, even under similar experimental conditions. Quantitative inheritance and environmental influence might have contributed toward upward or downward estimates of the carotenoids and may be another reason of poor relationships. More number of genic/ functional markers may give better insight to the problem investigated.

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