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Microsatellite-based genetic diversity in selected exotic and indigenous maize (*Zea mays* L.) inbred lines differing in total kernel carotenoids

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Abstract

A set of 24 diverse maize inbred lines, selected on the basis of their total kernel carotenoid concentration, were characterized using 36 microsatellite/simple sequence repeat markers distributed throughout the genome. A total of 140 polymorphic SSR alleles was detected, with a mean of four alleles per locus. The study led to the identification of eight informative SSR loci detecting 10 unique alleles. The average polymorphism information content was 0.55, with a range of 0.18 to 0.78. Genetic dissimilarity calculated using Jaccard's coefficient varied from 0.41 to 0.94 with a mean of 0.77. Cluster analysis using the unweighted pair group method with arithmetic mean showed four major clusters among the inbred lines. High degree of congruence was observed between clustering pattern and the pedigree data. Principal Coordinate Analysis (PCoA) also depicted the genetic diversity among the selected inbred lines. The study led to identification of genetically diverse and phenotypically contrasting inbred lines. These inbred lines can be potentially used in developing suitable mapping populations for QTL analysis, besides generating hybrid combinations with desirable kernel carotenoids.

Key words: Carotenoid, genetic diversity, heterotic group, maize, PIC, SSRs

Introduction

Carotenoids are the diverse group of colourful pigments that are synthesized in plastids such as chloroplasts, chromoplasts, and amyloplasts [1]. In photosynthetic organisms, they mainly function to prevent photo-oxidative damage of chlorophylls and contribute colours to many fruits and vegetables [2, 3]. Besides, these pigments serve various essential roles in human and animal nutrition; among the carotenoids, β-carotene is an important dietary precursor of vitamin A, deficiency of which leads to night blindness [4]. It is an important nutritional factor for proper immune function and reduces the risk of lung cancer, coronary heart disease, and some degenerative diseases [5]. It also acts as dietary antioxidant and scavenges free active molecules in the human body [6].

World Health Organization (WHO) estimates that vitamin A deficiency (VAD) affects an estimated 190 million pre-school children and 19 million pregnant women, mostly from Africa and South Asia [4]. Though various health interventions like fortification, supplementation and dietary diversification have been recommended, the increase of micronutrients in grains through breeding efforts popularly known as 'biofortification' is proposed as logical, sustainable and cost-effective means for providing required levels of micronutrients in the diets to alleviate malnutrition in humans [7, 8].

Maize serves as the staple food for hundreds of millions of people, especially in the tropical and subtropical areas of the developing world. Together with rice and wheat, maize provides at least 30% of the food calories for more than 4.5 billion people in 94 developing countries [9]. In India, 25% of the maize produce is used for human consumption, while 61% of the produce is utilized as poultry and animal feeds [10]. Maize shows tremendous variability for carotenoid concentration in kernel [11-13]. White kernel maize lacks carotenoid in the endosperm due to the presence of *yellow1* (*y1*) recessive allele in the homozygous

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condition. However, the dominant version of this allele, *Y1* produces enough carotenoids and makes the kernel yellow/orange in colour [14].

Assessing the extent of genetic variability is central to effective utilization of germplasm in the breeding programme. DNA markers, such as a microsatellite markers or SSRs, are particularly suited for genetic characterization and diversity studies, as compared to morphological markers [15-17]. The present investigation was aimed at assessing the genetic relationships among inbred lines for their effective utilization in breeding programme.

Materials and methods

Plant materials

A set of 24 maize inbred lines of exotic and indigenous origin were used in the present study for assessing the genetic relationship (Table 1). These inbreds were selected from a panel of 111 diverse inbred lines analyzed for variability for total carotenoids, as reported in a separate study [13].

Table 1. Details of maize inbred lines used in the study

S.No.	Inbred	Pedigree/source population	Institution	Total kernel carotenoids (µg/g)*
1	BAJIM-8-07	HAREC 95POOL-64-4	HAREC, Bajaura	10.6
2	BAJIM-8-10	HAREC 95POOL-202-2	HAREC, Bajaura	19.0
3	BLSB RIL-08	CM140 × CA01006 RIL-F2-8	IARI, New Delhi	30.6
4	BLSB RIL-92	CM140 × CA01006 RIL-F2-92	IARI, New Delhi	25.1
5	CM136	IPA 34-6-f-2-O	IARI, New Delhi	22.8
6	CM138	IPA 21-10-f-#-#-15	IARI, New Delhi	24.9
7	CM151	IPA 510-f-#	IARI, New Delhi	10.2
8	HKI1105	Cargil 633	HAU, Uchani	27.5
9	HKI586	CH 3	HAU, Uchani	6.5
10	NAI125	EV25-CD (Y) Alm-C7	UAS-Nagenahelli, Bangalore	25.8
11	NAI147	EV25-CD (Y) Alm-C7	UAS-Nagenahelli, Bangalore	10.9
12	SE547	Semi-exotic heterotic pool	PAU, Ludhiana	24.4
13	HPLET-03-08	[DTPYC9-F65-2-3-1-1-B-B x DTPYC9-F65-2-2-1-1-B-B]-3-4-2-B-B	CIMMYT-HarvestPlus	54.8
14	HPLET-03-09	[DTPYC9-F65-2-3-1-1-B-B x DTPYC9-F65-2-2-1-1-B-B]-6-3-3-B-B	CIMMYT-HarvestPlus	29.5
15	HPLET-03-10	[DTPYC9-F11-2-3-1-1-B-B x DTPYC9-F46-1-2-1-1-B]-B-1-1-B-B	CIMMYT-HarvestPlus	31.5
16	HPLET-03-23	MAS[206/312]-23-2-1-1-B-B-B/ [BETASYN]BC1-10-3-#-B-B	CIMMYT-HarvestPlus	15.3
17	HPLET-03-31	[CML197/N3//CML206]- x-32-1-4-B*5/ [BETASYN]BC1-4-4-4-1-B-B-B	CIMMYT-HarvestPlus	37.7
18	HPLET-03-32	P72c1xCML-297 x CL-02410-3-1-1-B-B-B	CIMMYT-HarvestPlus	37.4
19	HPLET-03-35	KUI carotenoid syn-FS17-3-1-B-B-B	CIMMYT-HarvestPlus	59.9
20	HPLET-03-36	KUI carotenoid syn-FS17-3-2-B-B-B	CIMMYT-HarvestPlus	67.3
21	HPLET-03-37	KUI carotenoid syn-FS25-3-2-B-B-B	CIMMYT-HarvestPlus	60.7
22	HPLET-03-41	Florida A plus Syn-FS2-2-1-B-B	CIMMYT-HarvestPlus	56.1
23	CML162	P25QPM	CIMMYT, Mexico	25.1
24	H16	Tuxpeno Sequa (white)	CIMMYT, Zimbabwe	1.3

*Data presented as per Sivaranjani *et al*. [13].

DNA isolation

Genomic DNA was isolated from three-week old young maize seedlings using CTAB-based DNA extraction method as per Saghai-Maroof *et al*. [18]. DNA concentration was estimated by λ uncut DNA (Bangalore Genei) in 1% agarose gel. The final concentration of genomic DNA was adjusted to10 ng/ µl by diluting the DNA in double distilled water.

SSR markers and PCR amplification

The SSR markers used in the present study were selected based on bin locations to cover the whole genome of maize. Primers for these loci were synthesized through Genex life science, using the sequence information available in the public domain (Maize GDB; http:/www.agron.missouri.edu). Out of 36 polymorphic markers selected for analysis of genetic diversity, 7 SSRs had di-repeat motif, 17 SSRs possessed tri-repeats and two SSRs had tetra-repeats (Table 2). Details of motif repeats of 10 SSRs are unknown. PCR amplification was carried out as per Nepolean *et al*. [19]. Amplified products were resolved in 3.5% SFR (Super Fine Resolution; Amersco, USA) agarose, in a 1X TBE buffer contained in horizontal gel electrophoresis system and visualization of DNA polymorphisms was done through a gel documentation system (Alpha Imager).

SSR data analysis

The SSR allele sizes were determined manually from the gel photographs by comparing the 100bp DNA ladder (Fermentas). Major allele frequency, gene diversity, unique alleles, rare alleles, and PIC values were computed using Power Marker 3.25 [20]. Pair-wise genetic dissimilarity coefficient of all possible genotype pairs was calculated using Jaccard's coefficient and hierarchical clustering was done using UPGMA (Unweighted Paired Group Method With Arithmetic Mean) algorithm. Principal Coordinate Analysis (PCoA) was also undertaken to study genetic relationships among the selected inbred lines. Both cluster analysis and the PCoA were carried out using DARwin 5.0 software [21].

Results and discussion

In total 140 alleles were detected across 36 SSR loci used in the study. The representative gels depicting SSR polymorphism among the inbreds is presented in Fig. 1. The number of alleles scored per SSR locus ranged from two (*umc1690*, *umc2298, bnlg278* and *umc1723*) to seven (*bnlg1803*, *bnlg1325, bnlg490* and *bnlg1185*) with a mean of nearly four. The PIC of the SSR loci was in the range of 0.18 (*umc2360*) to 0.78 (*bnlg1803*) with a mean of 0.55. SSR loci with di-repeat motif showed high level of polymorphism (mean PIC value of 0.68) as compared to loci with tri-repeat (0.49) or tetra-repeat (0.59), which confirms the observations from an earlier study [22]. Fourteen loci were found to have high PIC (>0.60) indicating the high discriminating power of SSR loci selected and the broad genetic variability of the genotypes analysed in this study (Table 2).

Eight SSR loci (*umc2360, umc2165, bnlg490, bnlg1178, bnlg1185, umc2296, umc1446* and *bnlg1803*) produced 10 unique alleles among nine specific inbreds (HPLET-03-41, HPLET-03-35, HKI1105, BAJIM-8-07, CM138, HPLET-03-10, BLSB RIL-08, CM136 and CM151). The occurrence of such a high number of unique alleles is possible due to the inclusion of diverse indigenous and exotic germplasm. Earlier studies [19, 23, 24] also reported occurrence of such unique alleles in selected maize inbreds. These unique alleles can be valuable in effectively differentiating genotypes and would aid in genetic fingerprinting analysis. Further, exploration of these unique alleles may also be useful in the context of marker-trait association [19]. The total number of rare alleles ($p<0.05$) detected among 24 selected geneotypes was 14; *umc2360, bnlg1325, umc2165* and *bnlg1178* were notable as having lowest allele frequencies among 140 SSR polymorphic alleles observed in this study.

Molecular marker-based estimation of heterozygosity among the inbred lines is also useful to assess the the purity of the seed lot [19, 23]. The average heterozygosity detected across 36 SSR loci was 0.09, suggesting attainment of a high degree of homozygosity in the inbred lines, and thereby high degree of genetic purity of selected inbreds. The heterozygosity detected at a few loci could be due to mutation at specific SSR locus, pollen and seed contamination, or amplification of similar sequences from different genomic regions due to duplication [25]. In inbred lines derived through conventional breeding (as compared to doubled haploids), residual heterozygosity is not uncommon.

The average major allele frequency was 0.49, with a range of 0.30 (250 bp for *bnlg1803*) to 0.90 (110bp for *umc2360*). The major allele frequency with low value is indicative of highly diverse locus among the selected genotypes [19]. Gene diversity is defined as the probability that two randomly chosen alleles

Table 2. SSR data and summary statistics of the genotyping assay for the maize inbreds

S.No.	SSR locus	Bin location	SSR motif	Major allele frequency	Allele detected	Gene diversity	Hetero-zygosity	PIC
1	*bnlg1178*	1.02	(AG)16	0.39	6	0.74	0.14	0.70
2	*bnlg1803*	1.02	(AG)15	0.30	7	0.81	0.22	0.78
3	*umc2083*	1.06	(CGG)7	0.65	3	0.52	0.08	0.47
4	*umc1446*	1.08	(TAA)7	0.42	5	0.70	0.13	0.64
5	*bnlg381*	2.04	-	0.56	4	0.61	0.04	0.57
6	*phi083*	2.04	AGCT	0.50	3	0.61	0.00	0.54
7	*umc2077*	2.06	(AGC)4	0.39	5	0.75	0.00	0.72
8	*bnlg1325*	3.03	(AG)18	0.33	7	0.76	0.08	0.73
9	*umc1690*	3.07	(GCA)4	0.54	2	0.50	0.17	0.37
10	*umc1757*	4.01	(TCC)7	0.50	3	0.61	0.08	0.54
11	*phi021*	4.03	-	0.73	3	0.42	0.13	0.37
12	*bnlg490*	4.04	-	0.35	7	0.79	0.00	0.76
13	*umc2139*	4.09	(GCC)4	0.65	3	0.50	0.17	0.43
14	*umc2360*	4.09	(GCC)4	0.90	3	0.19	0.04	0.18
15	*bnlg143*	5.01	-	0.50	3	0.61	0.04	0.54
16	*umc2296*	5.03	-	0.35	5	0.75	0.21	0.71
17	*umc2298*	5.03	(GCG)4	0.63	2	0.47	0.00	0.36
18	*phi113*	5.04	GTCT	0.38	4	0.70	0.00	0.64
19	*bnlg278*	5.05/5.06	-	0.58	2	0.49	0.00	0.37
20	*phi087*	5.06	ACC	0.39	3	0.65	0.04	0.58
21	*umc1723*	6.02	(CTT)6	0.88	2	0.22	0.00	0.19
22	*bnlg1014*	6.04	(AG)14	0.54	3	0.60	0.00	0.53
23	*bnlg1702*	6.05	(AG)17	0.44	3	0.63	0.04	0.56
24	*umc2165*	6.07	(TTC)12	0.33	6	0.75	0.13	0.71
25	*umc2364*	7.01	(GGA)7	0.38	3	0.66	0.00	0.59
26	*phi034*	7.02	CCT	0.54	3	0.56	0.17	0.47
27	*umc1831*	7.02	(AG)8	0.38	5	0.72	0.13	0.68
28	*umc2334*	7.06	(GGA)4	0.42	4	0.69	0.17	0.64
29	*umc1872*	8.02	(GCA)6	0.69	3	0.48	0.04	0.43
30	*umc1562*	8.05	-	0.48	3	0.62	0.04	0.55
31	*bnlg240*	8.06	-	0.35	4	0.69	0.04	0.63
32	*phi028*	9.01	-	0.50	3	0.57	0.25	0.48
33	*bnlg619*	9.07	-	0.35	4	0.69	0.08	0.63
34	*umc2017*	10.03	(CAA)4	0.50	4	0.63	0.21	0.57
35	*bnlg1185*	10.07	(AG)24	0.31	7	0.80	0.17	0.77
36	*umc2021*	10.07	(TGG)4	0.48	3	0.59	0.04	0.50
	Mean			0.49	3.89	0.61	0.09	0.55

*PIC: Polymorphism information content

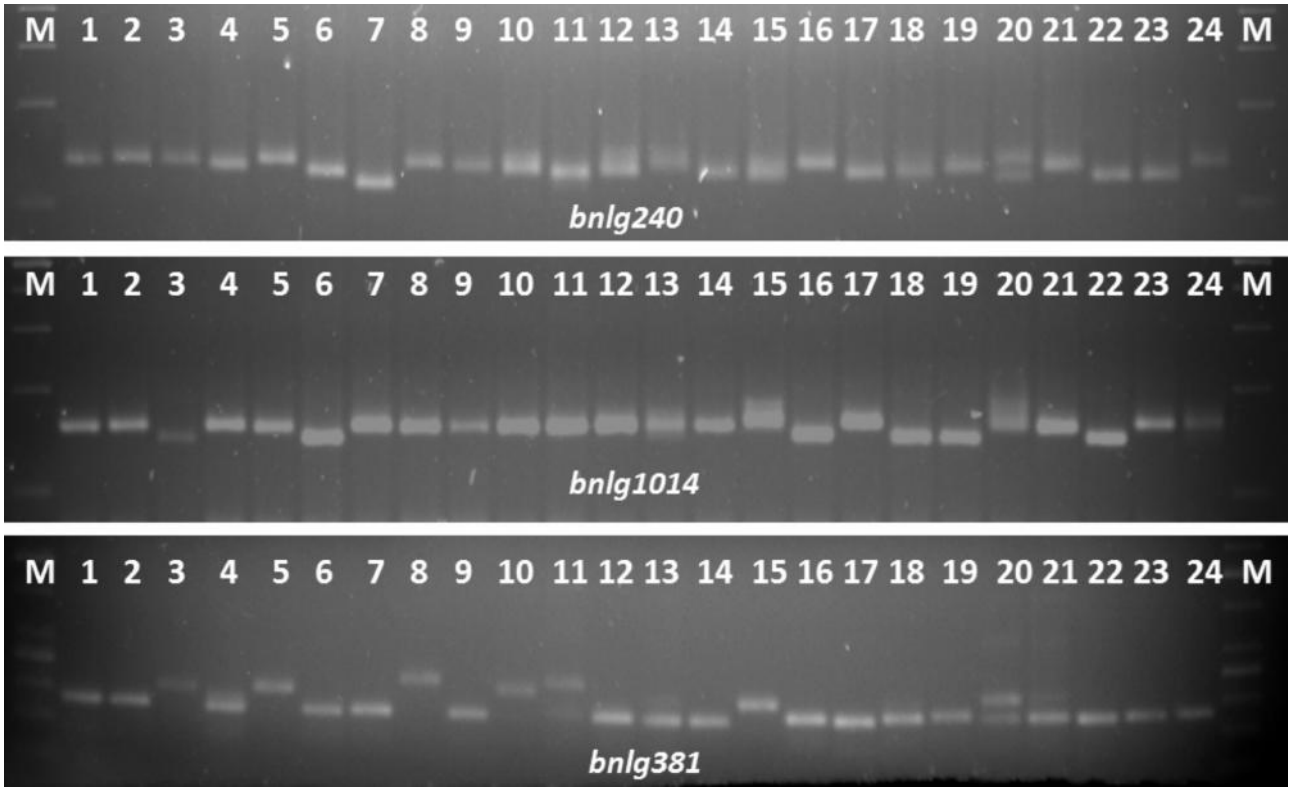

Fig. 1. SSR polymorphism among 24 selected maize inbreds. M: marker, 1: SE547, 2: HKI586, 3: CML162, 4: BAJIM-8-10, 5: HPLET-03-23, 6: HPLET-03-08, 7: H16, 8: HPLET-03-41, 9: HPLET-03-35, 10: HKI1105, 11: BAJIM-8-07, 12: HPLET-03-32, 13: CM138, 14: HPLET-03-37, 15: HPLET-03-10, 16: BLSB RIL-92, 17: HPLET-03-36, 18: HPLET-03-09; 19: BLSB RIL-08, 20: CM136, 21: HPLET-03-31, 22: CM151, 23: NAI125, 24: NAI147

from the population are different [20]. The highest gene diversity obtained was 0.81 (*bnlg1803*), while the lowest was 0.19 (*umc2360*) with an average of 0.61.

Genetic dissimilarity coefficients ranged from 0.41 (HPLET-03-36 and HPLET-03-35) to 0.94 (HPELT-03-09 and HKI1105) with average dissimilarity being 0.77, thereby indicating the presence of high genetic diversity among the maize genotypes analyzed. Dissimilarity matrix generated using Jaccard's coefficient was used to construct a tree using the UPGMA algorithm in hierarchical clustering method. It grouped the 24 genotypes into four major clusters (Fig. 2). Cluster A comprised of nine inbred lines, and could be further grouped into three sub clusters where, sub-cluster A1 comprised of HPLET-03-35, HPLET-03-36 and HPLET-03-37, while CM151 and CML162 were found in sub-cluster A2. In case of sub-cluster A3, four inbreds (BLSB RIL-92, HKI586, HPLET-03-31 and BLSB RIL-08) clustered together. Cluster B was the smallest cluster having two inbreds lines (H16 and SE547). In contrast, Cluster C emerged as the largest group having ten inbreds from both

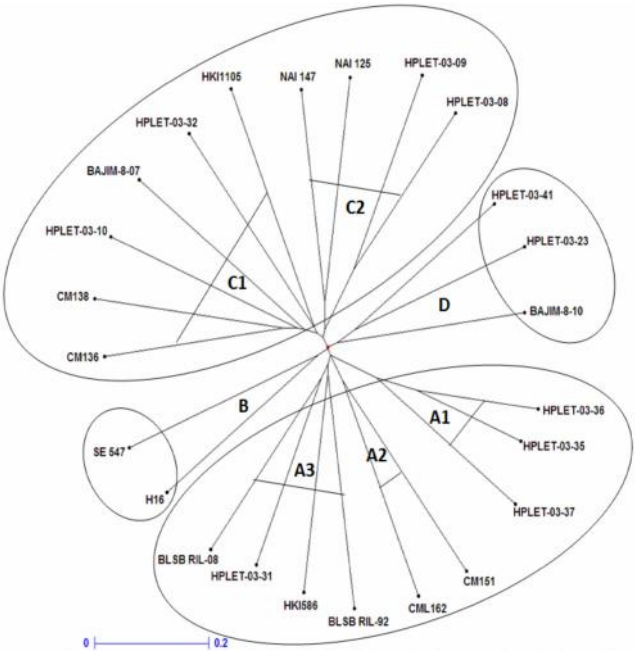

Fig. 2. Cluster analysis among 24 maize inbreds depicting their genetic relationship

indegenous and exotic origin, and was further sub-divided into two sub-groups. Sub-cluster C1 consisted of CM136, CM138, BAJIM-8-07 and HKI1105 of Indian origin, and HPLET-03-10 and HPLET-03-32 of CIMMYT-HarvestPlus origin. Sub-cluster C2 had four inbred lines of which two were of Indian origin (NAI147 and NA125) and two were from CIMMYT-HarvestPlus (HPLET-03-09 and HPLET-03-08). Cluster D consisted of two CIMMYT-HarvestPlus (HPLET-03-41 and HPLET-03-23) and one Indian origin inbred (BAJIM-8-10). Interestingly, clustering pattern depicted robust congruence with pedigree data (Table 1). For example, NAI147 and NAI125, developed at University of Agricultural Sciences, Naganahalli could be grouped together. Similarly, HPLET-03-08 and HPLET-03-09 in sub-cluster C2, HPLET-03-35 and HPLET-03-36 in sub-cluster A1 and BLSB RIL-92 and BLSB RIL-08 in sub-cluster A3, also shared common pedigree and are grouped together (Table 1). Das and Singh [26] characterized 25 inbred lines varying for total carotenoids using 12 SSR markers and grouped them in four distinct clusters.

The genetic relationships as depicted by cluster analysis were further reconfirmed by Principal Coordinate Analysis (PCoA). The PCoA plot (Fig. 3) shows both indigenous and exotic lines distributed in all quadrangles, thereby suggesting wide genetic variability among these inbreds. NAI147 and NAI125 of Indian origin could be placed in the same quadrangle due to their similar pedigree. Exotic inbreds, HPLET-03-8 and HPLET-03-9 also showed similar trend. The PCoA analysis also depicted that HPLET-03-36 and HPLET-03-37 (top right qualdrangle), HPLET-03-08, HPLET-03-09 and BAJIM-8-07 (top left quadrangle) and NAI125, NAI147 and CM136 (left bottom quadrangle) were placed quite far from the rest of the inbreds, indicating their genetic distinctness.

All the 24 lines were found to be agronomically suitable (results not presented here), besides showing significant variation in total kernel carotenoid concentration (Table 1). Systematic crosses among selected lines based on the available information could be useful in further genetic analysis as well as in biofortification program. Firstly, genetically diverse parents with contrasting total carotenoid concentration can be crossed to generate $F_{2:3}$ and/or RIL mapping population to identify QTLs governing the accumulation of total kernel carotenoids. HPLET-03-35, HPLET-03-36 and HPLET-03-37 having a higher concentration of total carotenoids can be crossed with inbreds with low carotenoids such as NAI147 and BAJIM-8-07, to generate such mapping populations (Fig. 2, Table 1).

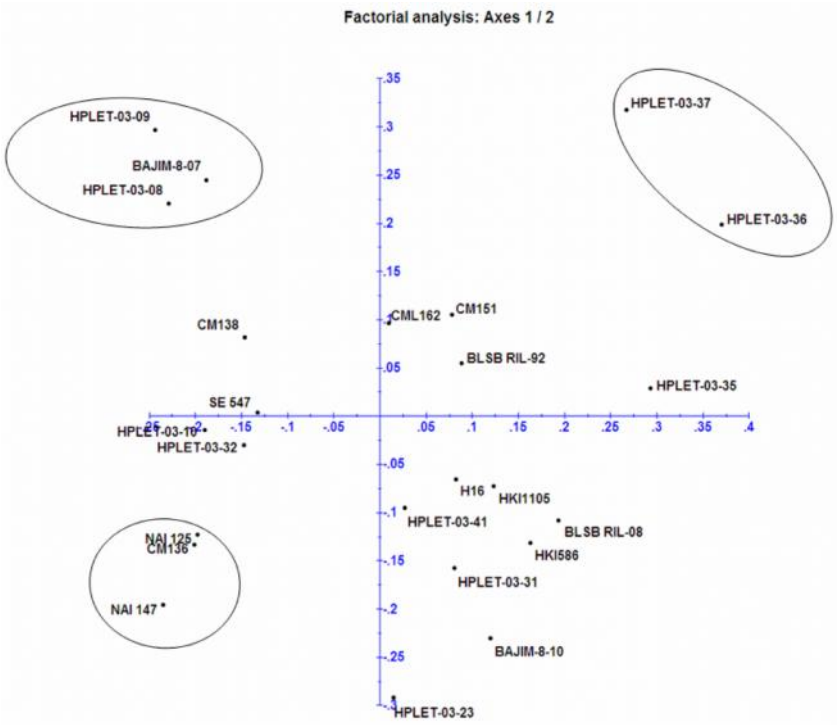

Fig. 3. Principal coordinate analysis (PCoA) among 24 maize inbreds using 36 SSR markers

Besides, HPLET-03-8 x HKI586, HPLET-03-8 x CM151, HPLET-03-41 x HKI586 and HPLET-03-41 x CM151 can also be attempted for the given purpose. Although the carotenoid biosynthetic pathway in maize is well-characterised and genes such as *psy, crtRB1* and *lcyE* have been identified to play important roles in the synthesis of carotenoids in maize kernel [11, 14, 27, 28], but such an analysis could potentially reveal other possible genes/loci influencing kernel carotenoid concentration in maize [29].

Secondly, enhancing the genetic base of elite germplasm through introgression of exotic germplasm is important for the heterotic potential [30]. Based on the genetic relationships and high total kernel carotenoid concentration, the following possible heterotic combinations could be potentially identified through the present study: HPLET-03-36 x HPLET-03-8, HPLET-03-36 x HPLET-03-41, HPLET-03-37 x HPLET-03-8, HPLET-03-37 x HPLET-03-41, HPLET-03-35 x HPLET-03-8, HPLET-03-35 x HPLET-03-41, HPLET-03-8 x HPLET-03-41. It is important to note that none of the inbreds of Indian origin showed high total kernel carotenoid; however, inbreds with moderate total kernel carotenoid concentration can be crossed with diverse CIMMYT-HarvestPlus inbreds with high total kernel carotenoid concentration. Crosses such as, BLSBRIL-8 x HPLET-03-41, BLSBRIL-8 x HPLET-03-8, HKI1105 x HPLET-03-41, HKI1105 x HPLET-03-35, HKI1105 x HPLET-03-36 and HKI1105 x HPLET-03-37 could be attempted to develop hybrids with a desirable concentration of carotenoids.

The present study thus dealt with the estimation of genetic diversity among the selected set of inbred lines from Indian maize breeding programme and their comparison with lines developed by CIMMYT-HarvesPlus. Cluster analysis and PCoA revealed high degree of genetic variability among the selected set of indigenous and exotic maize inbred lines differing in total kernel carotenoid concentrations. The study led to the identification of suitable cross combinations that could be planned and generated for further use in genetic and breeding strategies pertaining to improvement of kernel carotenoids in maize.

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