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Characterization of maize genotypes using microsatellite markers associated with QTLs for kernel iron and zinc

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Crop genetic resources rich in Fe and Zn provide sustainable and cost-effective solution to alleviate micronutrient malnutrition. Maize being the leading staple crop assumes great significance as a target crop for biofortification. We report here wide genetic variation for kernel Fe and Zn among 20 diverse maize inbreds lines, majority of which were bred for quality protein maize (QPM) and provitamin-A. Kernel Fe ranged from 30.0 - 46.13 mg/kg, while kernel Zn ranged from 18.68-39.56 mg/kg. Moderate but positive correlation was observed between the micronutrients. Characterization using 25 Single sequence repeats (SSRs) linked to QTLs for kernel Fe produced 58 alleles. Similarly, 86 alleles were identified from 35 SSRs linked to QTLs for kernel Zn. One unique allele for kernel Fe and three unique alleles for kernel Zn were identified. The mean polymorphic information content (PIC) was 0.40 for both kernel Fe and Zn. Jaccard's dissimilarity coefficients varied from 0.25 - 0.91 with a mean of 0.58 for kernel-Fe while 0.27- 0.88 with a mean of 0.57 for kernel Zn. Principal coordinate analysis depicted diversity of inbreds. Cluster analysis grouped the inbreds into three major clusters for both kernel Fe and Zn. Potential cross combinations have been proposed to develop micronutrient rich hybrids and novel inbreds with higher Fe and Zn. The information generated here would help the maize biofortification programme to develop nutritionally enriched hybrids.

Keywords: QPM, QTL, kernel Fe, kernel Zn micronutrient, SSR, biofortification,

Introduction

Micronutrient malnutrition has emerged as one of the alarming problems affecting an estimated two billion people worldwide[1]. The problem is more prevalent in the under-developed and developing world where resource poor people depend upon cereal-based diets that are inherently deficient in micronutrients[2]. Of the 667 million children worldwide under the age of five, 159 million are stunted, while 50 million do not weigh enough for their height[3]. As per global food policy report by IFPRI, South Asia is home to more than 35% of the world's poor, and 21.9% of the population of India lives in poverty, and thus vulnerable to various health problems. Malnutrition poses severe socio-economic loss that amounts to 11% of the annual gross domestic product (GDP) of Asia and Africa, and India loses over $12 billion annually in GDP to micronutrient deficiencies (www.harvestplus.org).

Among various micronutrients, iron (Fe) and zinc (Zn) deficiencies in humans are most pronounced worldwide[4]. Humans require Fe for basic cellular functions and proper functioning of the muscle, brain and red blood cells. Zn plays a crucial role for more than 300 enzymes in the human body for the synthesis and degradation of carbohydrates, lipids, proteins and nucleic acids[5]. Iron deficiency affects cognitive development, growth, reproductive performance and work productivity, while inadequate consumption of Zn leads to depression and psychosis, impaired growth and development besides affecting immune system[6]. It is estimated that over 60% of the world's population are Fe-deficient while 30% are affected due to Zn deficiency[7]. In India, 70% and 48% of children under five are estimated to be deficient in Fe and Zn, respectively.

Since, Fe and Zn cannot be synthesized in human body; they must be made available through diet. Various methods such as 'food fortification', 'supplementation' and 'dietary diversification' are practiced all over the world for alleviating the micronutrient deficiencies.

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'Biofortification', a process in which micronutrient density in crops is increased by plant breeding, is proposed as a sustainable and cost-effective mean for providing the required levels of micronutrients in pure form[7]. According to Global Nutrition Report, it has been estimated that alleviating malnutrition is one of the most cost-effective steps, and every $1 invested in proven nutrition programme offers benefits worth $16. Thus efforts directed towards providing the balanced and nutritious food assumes great significance[8].

Maize occupies an important position in the world economy, and also serves as important source of food. Together with rice and wheat, it provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries[9]. In India, maize is an important cereal too and provides vital source of food and energy to large human populations[10]. Significant variation for grain Fe and Zn in maize germplasm has been reported worldwide. Understanding the genetic relationships among inbreds is one of the most important steps for their effective utilization in the breeding programme[11]. Over the past few years, a number of quantitative trait loci (QTL) that are responsible for the accumulation of kernel Fe and Zn in maize kernels have been reported[12-16]. So far large number of studies have characterized maize inbreds for Fe and Zn by analyzing their accumulation in kernel[17-23]. Very few reports on molecular characterization of inbreds for Fe and Zn in maize are available, where SSRs distributed throughout the genome were used to understand their genetic relationships[24-25]. So far no information on characterization of inbreds using QTLs for Fe and Zn is available in maize. In the present investigation a set of diverse quality protein maize (QPM) and provitamin-A rich maize inbreds were analyzed for accumulation of Fe and Zn in kernel, and subsequently characterized using microsatellite markers linked to QTLs for Fe and Zn to (i) assess the genetic relationship among inbred lines; and (ii) identify potential cross combinations to develop micronutrient rich biofortified maize genotypes.

Materials and Methods

Plant Materials

A set of 20 maize diverse inbreds including six provitamin-A rich, eight QPM and three with both provitamin-A and QPM, were evaluated during *kharif* 2015 at Experimental Farm, ICAR-Indian Agricultural Research Institute, New Delhi. Majority of the inbred lines were developed through marker-assisted selection approach. Beside, three normal elite inbreds from CIMMYT, Mexico, and national system, were also taken. The inbreds were analyzed in a randomized complete block design (RCBD) with three replications per entry and one row per replication. Plant-to-plant spacing of 20 cm and row-to-row spacing of 75 cm were maintained. Standard agronomic practices were followed for raising and maintenance of the plants. Three random plants from each row were selfed for analyses for kernel Fe and Zn. V345-PV-A and V345-PV-B, two sister inbred lines were taken as internal check for the analyses on genetic relationship.

Biochemical Estimation of Kernel Fe and Zn

Three selfed ears per genotype were hand-harvested with husk and dried under clean shade to reduce post harvest grain moisture concentration to ~14%. Care was taken so that the grain does not come in contact of hand, soil or any metal objects. Representative grain samples were drawn in triplicate by quartering method and the individual samples were ground in to fine powder using iron free Cyclotech Sample Mill. The flour sample (0.5 g) was digested with di-acid mixture in a ratio 10:4 (HNO_3: $HClO_4$) using standard protocol[26-27]. The kernel Fe and Zn concentration in the samples were analyzed using an Atomic Absorption Spectrophotometer (AAS-Perkin Elmer) at ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, Uttarakhand, India.

SSR Markers and PCR Amplification

The leaf samples were taken from three randomly selected plants of each inbred (3 weeks old) and bulked to extract DNA. The genomic DNA was extracted from bulked leaf sample using modified CTAB procedure[28]. DNA concentration was measured by comparing an aliquot of the extracted sample with standard DNA of known concentration using gel electrophoresis. The final concentration of extracted DNA was adjusted to 25 ng/μL with 1X TE buffer (pH 8.0) and stored at 4°C for future use. A total 54 SSR markers including 27 markers linked to the QTLs for kernel-Fe, and 35 SSR markers associated with the QTLs for kernel-Zn, reported in different QTL mapping studies were used for molecular characterization of 20 inbred lines of maize Information of SSRs reported by Jaiswal[29] was obtained from VPKAS, Almora. The primer information for the selected SSRs was obtained from

www.maizegdb.org. Approximately 25 ng of DNA was used as template for PCR in a 20 µl reaction volume in Applied Biosystems (Veriti 96 well Thermal Cycler). PCR cycling consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 1 min, 55-65°C (based on Tm values of different SSR primers) for 1 min and 72°C for 1 min. A final extension step at 72°C for 7 min was followed by termination of the cycle at 4°C. The amplified products were resolved on 4.0% agarose gel (Lonza, Rockland, ME USA) using gel electrophoresis system (Scie-Plus, Axygen) at 120 volts for 3 to 4 hours.

SSR Data Analysis

The allele size was determined by comparing the 100 bp DNA ladder. Gene diversity, major allele frequency, unique and rare alleles, and polymorphism information content (PIC) values were computed using Power Marker 3.25[30] (Table 1). Genetic dissimilarity was calculated using Jaccard's coefficient. Cluster analysis following unweighted neighbour-joining method and principal coordinate analysis (PCoA) were undertaken using DARwin6.0[31].

Results

Genetic Variation for Kernel Fe and Zn

Significant genetic variation for both the kernel Fe and Zn concentration among the diverse maize inbred lines were observed (Table 2). The kernel Fe concentration ranged from 30.0-46.13 mg/kg, while kernel Zn ranged from 18.68-39.56 mg/kg (Table 1).

Table 1 — Details of maize inbreds analyzed for kernel Fe and Zn

S. no.	Genotypes	Type*	Institution	Fe (mg/kg)	Zn (mg/kg)
1.	HPLET-03-3	ProA	HarvestPlus	31.00	31.22
2.	HP-19-01	ProA	HarvestPlus	34.83	28.47
3.	V335-PV	ProA	IARI, New Delhi	34.05	39.56
4.	V345-PV-A	ProA	IARI, New Delhi	36.34	31.19
5.	V345-PV-B	ProA	IARI, New Delhi	36.58	33.82
6.	HKI1105-PV	ProA	IARI, New Delhi	37.84	29.40
7.	HKI1105-Q	QPM	IARI, New Delhi	31.61	28.76
8.	HKI1128-Q	QPM	IARI, New Delhi	32.46	28.54
9.	HKI323-Q	QPM	IARI, New Delhi	33.76	22.11
10.	CM150-Q	QPM	IARI, New Delhi	32.39	23.94
11.	CM151-Q	QPM	IARI, New Delhi	32.45	18.68
12.	MGUQ-1-opaque	QPM	IARI, New Delhi	34.60	38.51
13.	MGUQ-2-mod	QPM	IARI, New Delhi	33.76	30.14
14.	HKI161	QPM	CSSHAU, Uchani	30.00	23.31
15.	VQL1-PV	QPM + ProA	IARI, New Delhi	34.54	33.88
16.	VQL2-PV	QPM + ProA	IARI, New Delhi	35.40	22.98
17.	HKI1105-Q+PV	QPM + ProA	IARI, New Delhi	34.64	25.60
18.	CM501	Normal	IIMR, New Delhi	46.13	38.36
19.	CML319	Normal	CIMMYT, Mexico	33.68	30.27
20.	CML336	Normal	CIMMYT, Mexico	32.32	29.66
		Mean		34.42	29.42
		CD (5%)		3.83	2.12

* ProA: provitamin-A, QPM: Quality protein maize, CD: Critical difference

Table 2 —Analysis of variance for kernel Fe and Zn in maize inbreds

S. no.	Sources of variation	df	Fe		Zn	
			MS	Prob.	MS	Prob.
1.	Genotypes	19	34.00**	0.00000	95.78**	0.00000
2.	Replication	2	2.47	0.63472	0.38	0.79248
3.	Error	38	5.37	-	1.64	-
4.	Total	59	14.49	-	31.91	-

**Significant at 0.01, df: degrees of freedom, MS: Mean Square, Prob.: Probability

The mean kernel-Fe was 34.42 mg/kg and the same for Zn was 29.42 mg/kg. CM501 (46.13 mg/kg) was identified as the best inbred for Fe, followed by HKI1105-PV (37.84 mg/kg), V345-PV-B (36.58 mg/kg,), V345-PV-A (36.34 mg/kg) and VQL2-PV (35.40 mg/kg). In case of Zn, V335-PV (39.56 mg/kg) was the most promising genotype. Further, MGUQ-1-opaque (38.51 mg/kg), CM501 (38.36 mg/kg), VQL1-PV (33.88 mg/kg), V345-PV-B (33.82 mg/kg), HPLET-03-3 (31.22 mg/kg), V345-PV-A (31.19 mg/kg), CML319 (30.27 mg/kg) and MGUQ-2-mod (30.14 mg/kg) possessed >30 mg/kg of Zn (Table 2). Considering both the minerals, CM501, V335-PV, MGUQ-1-opaque, VQL1-PV, V345-PV-B, V345-PV-A and HPLET-03-3 were identified as the top most genotypes. Kernel-Fe and -Zn showed significant positive correlation (r = 0.47*). The Fe and Zn content of the soil were 4.531 ppm and 0.892 ppm at 0-15 cm depth, while it was 4.123 ppm and 0.821 ppm at 15-30 cm depth.

SSR Polymorphism

Of the 27 SSRs linked to kernel-Fe, *umc1888* and *umc1620* were monomorphic. A total of 58 alleles were detected across 25 SSR loci for kernel-Fe. The allele numbers varied from two to five with an average of 2.32 alleles per locus. The PIC value for SSR linked to Fe ranged from 0.20 (*bnlg339*) to 0.66 (*umc1673*) with a mean of 0.40. Similarly, a total of 86 alleles were detected across 35 SSR loci associated with kernel-Zn with an average 2.46 alleles per locus. The allele numbers varied from two to four per locus. The PIC value for kernel-Zn ranged from 0.10 (*bnlg2336*) to 0.59 (*bnlg1288*) with a mean of 0.40. Across inbreds, Jaccard's dissimilarity coefficients varied from 0.25-0.91 with a mean of 0.58 for kernel-Fe and 0.27-0.88 with a mean of 0.57 for kernel-Zn. Among 54 SSRs used in the study, twenty five loci had di-repeat motif, ten loci had tri-repeat, five loci had tetra-repeat motif, four loci had penta-repeat motif and three loci had hexa-repeat motif (Table 3).

Table 3 — Primer details and summary statistics of genotyping assay undertaken among inbreds

S. no.	Markers	Bin	Repeat	Major allele freq.	No. of allele	Gene Diversity	Hetero -zygosity	PIC	Associated trait	References
1.	*bnlg1884*	1.05	$(AG)_{13}$	0.47	4	0.59	0.00	0.51	Fe	Gu *et al.*[16]
2.	*umc1919*	1.06	$(CT)_8$	0.90	2	0.18	0.00	0.16	Zn	Qin *et al.*[13]
3.	*umc1754*	1.06	$(CGAT)_5$	0.56	2	0.49	0.06	0.37	Fe and Zn	Gu *et al.*[16]
4.	*umc1335*	1.06	$(AG)_{24}$	0.68	3	0.46	0.00	0.39	Zn	Gu *et al.*[16]
5.	*umc2586*	1.08	(CA)	0.65	2	0.46	0.00	0.35	Zn	Qin *et al.*[13]
6.	*umc1118*	1.11	$(GAGCA)_4$	0.50	3	0.61	0.00	0.53	Zn	Qin *et al.*[13]
7.	*umc1542*	2.02	$(AG)_{10}$	0.69	3	0.46	0.00	0.40	Fe	Qin *et al.*[13]
8.	*umc1518*	2.02	$(TAT)_5$	0.47	3	0.63	0.00	0.56	Fe	Gu *et al.*[16]
9.	*umc1042*	2.07	$(GA)_{17}$	0.68	2	0.43	0.00	0.34	Fe	Qin *et al.*[13]
10.	*umc1230*	2.08	$(TAA)_8$	0.56	3	0.54	0.00	0.45	Fe and Zn	Qin *et al.*[13]
11.	*umc1464*	2.08	$(CCA)_6$	0.60	2	0.48	0.00	0.36	Fe and Zn	Qin *et al.*[13]
12.	*umc1386*	3.04	$(CTCC)_4$	0.63	2	0.47	0.00	0.36	Zn	Qin *et al.*[13]
13.	*umc1504*	3.04	$(AAAAG)_5$	0.79	2	0.33	0.00	0.28	Zn	Qin *et al.*[13]
14.	*nc004*	4.03	(AG)	0.85	3	0.27	0.00	0.25	Fe	Qin *et al.*[13]
15.	*bnlg1217*	4.05	$(AG)_{33}$	0.61	2	0.48	0.00	0.36	Fe	Qin *et al.*[13]
16.	*umc1329*	4.06	$(GCC)_7$	0.55	2	0.50	0.00	0.37	Fe	Qin *et al.*[13]
17.	*umc1620*	4.07	$(TTC)_4$	1.00	1	0.00	0.00	0.00	Fe	Gu *et al.*[16]
18.	*umc1194*	4.07	(GGCC)	0.56	2	0.49	0.00	0.37	Fe and Zn	Gu *et al.*[16]
19.	*phi113*	5.03	(GTCT)	0.43	3	0.64	0.05	0.56	Zn	Gu *et al.*[16]
20.	*umc1705*	5.03	$(AG)_{28}$	0.61	2	0.48	0.00	0.36	Zn	Jaiswal[29]
21.	*umc1990*	5.04	-	0.50	2	0.50	0.00	0.38	Zn	Gu *et al.*[16]
22.	*bnlg2323*	5.04	$(AG)_{25}$	0.55	2	0.50	0.00	0.37	Zn	Gu *et al.*[16]
23.	*bnlg278*	5.05	-	0.40	3	0.65	0.05	0.57	Zn	Gu *et al.*[16]
24.	*umc1155*	5.05	$(AG)_{20}$	0.58	3	0.55	0.00	0.48	Zn	Jaiswal[29]
25.	*umc2306*	5.06	$(TATATA)_4$	0.72	2	0.40	0.00	0.32	Zn	Gu *et al.*[16]
26.	*mmc0481*	5.06	$(GA)_{29}$	0.53	4	0.61	0.00	0.54	Zn	Qin *et al.*[13]

(*Contd*.)

Table 3 — Primer details and summary statistics of genotyping assay undertaken among inbreds (Contd.)

S. no.	Markers	Bin	Repeat	Major allele freq.	No. of allele	Gene Diversity	Hetero -zygosity	PIC	Associated trait	References
27.	*umc1019*	5.06	$(CT)_{17}$	0.55	4	0.63	0.00	0.58	Zn	Gu *et al.*[16]
28.	*bnlg1306*	5.07	$(AG)_{21}$	0.58	5	0.61	0.00	0.58	Zn	Qin *et al.*[13]
29.	*umc2319*	6.05	$(GAGGAG)_5$	0.75	2	0.38	0.00	0.30	Zn	Qin *et al.*[13]
30.	*bnlg1732*	6.05	$(AG)_{15}$	0.67	2	0.44	0.00	0.35	Zn	Qin *et al.*[13]
31.	*bnlg1740*	6.07	$(AG)_{21}$	0.75	2	0.38	0.00	0.30	Zn	Gu *et al.*[16]
32.	*umc2059*	6.08	$(CAG)_8$	0.68	2	0.43	0.00	0.34	Zn	Gu *et al.*[16]
33.	*mmc0171*	7.00	$(GA)_{29}$	0.50	2	0.50	0.00	0.38	Fe	Gu *et al.*[16]
34.	*umc1016*	7.02	$(CT)_{25}$	0.67	2	0.44	0.00	0.35	Fe	Qin *et al.*[13]
35.	*umc1112*	7.03	$(TC)_6$	0.50	3	0.59	0.00	0.50	Zn	Qin *et al.*[13]
36.	*bnlg339*	7.03	-	0.87	2	0.23	0.16	0.20	Fe and Zn	Gu *et al.*[16]
37.	*umc1015*	7.03	$(GA)_{45}$	0.63	4	0.55	0.00	0.52	Zn	Jaiswal[29]
38.	*umc1888*	7.03	$(ATA)_6$	1.00	1	0.00	0.00	0.00	Fe	Gu *et al.*[16]
39.	*umc1125*	7.04	$(CTCG)_5$	0.58	2	0.49	0.15	0.37	Fe and Zn	Qin *et al.*[13]
40.	*umc1944*	7.04	-	0.45	3	0.65	0.00	0.57	Zn	Qin *et al.*[13]
41.	*bnlg1056*	8.08	$(AG)_{16}$	0.75	2	0.38	0.00	0.30	Fe	Gu *et al.*[16]
42.	*umc1673*	8.08	$(TCC)_5$	0.40	5	0.71	0.05	0.66	Fe	Gu *et al.*[16]
43.	*umc1957*	9.00	-	0.53	2	0.50	0.00	0.37	Fe	Qin *et al.*[13]
44.	*bnlg1288*	9.01	$(AG)_{15}$	0.37	3	0.66	0.00	0.59	Fe and Zn	Qin *et al.*[13]
45.	*dup ssr 29*	9.07	$(GA)_{24}$	0.63	2	0.47	0.11	0.36	Fe and Zn	Qin *et al.*[13]
46.	*umc1962*	10.03	-	0.68	2	0.43	0.00	0.34	Fe	Qin *et al.*[13]
47.	*umc1938*	10.03	-	0.80	2	0.32	0.00	0.27	Fe	Qin *et al.*[13]
48.	*umc1345*	10.03	$(GCC)_4$	0.68	2	0.43	0.00	0.34	Zn	Gu *et al.*[16]
49.	*umc1336*	10.03	$(ACCAG)_4$	0.58	2	0.49	0.06	0.37	Zn	Gu *et al.*[16]
50.	*bnlg2336*	10.04	$(AG)_{16}$	0.94	2	0.11	0.00	0.10	Zn	Qin *et al.*[13]
51.	*umc1196*	10.07	(CACACG)	0.40	3	0.66	0.00	0.59	Fe	Qin *et al.*[13]; Jaiswal[29]
52.	*umc2021*	10.07	$(TGG)_4$	0.56	3	0.54	0.06	0.44	Fe	Qin *et al.*[13]
53.	*umc1556*	10.07	$(ATTTA)_6$	0.43	4	0.64	0.05	0.57	Zn	Gu *et al.*[16]
54.	*bnlg1839*	10.07	$(AG)_{24}$	0.61	2	0.48	0.00	0.36	Fe	Jaiswal[29]

PIC: Polymorphism Information Content

PIC (0.66) value of tri-repeat based SSR loci was higher than the other higher repeat motifs (0.10-0.59). The present study produced one unique allele for kernel-Fe by *nc004* in the genotypes VQL2-PV. Three unique alleles were also identified for kernel-Zn by *umc1556, umc1335* and *bnlg2336* in VQL2-PV, CM501 and CML319, respectively. No rare allele ($P < 0.01$) was detected for SSRs linked to both Fe and Zn. Allele present in maximum number of screened inbreds is described as the major allele. In the present study the average major allele frequency for kernel-Fe was 0.60, with a range of 0.37 (*bnlg1288*) to 0.87 (*bnlg339*). For kernel-Zn, major allele frequency ranged 0.37 (*bnlg1288*) to 0.94 (*bnlg2336*) with an average of 0.61. Gene diversity varied from 0.23 (*bnlg339*) to 0.71 (*umc1673*) with an average of 0.49, while for kernel-Zn, it ranged from 0.11 (*bnlg2336*) to 0.66 (*bnlg1288*) with an average of 0.49. The heterozygosity observed among the SSR loci varied from 0.00 to 0.16 for both kernel Fe and Zn, with a mean of 0.02 (Table 3).

Genetic Relationships

Cluster analysis of 20 inbred lines for both the kernel Fe and Zn were conducted based on genetic dissimilarities from SSR data using UPGMA method (Fig. 1). The genetic dissimilarity coefficient varied from 0.25 (V345-PV-A and V345-PV-B) to 0.91 (HKI1105 -Q+PV and MGUQ-1-opaque) with a mean value of 0.58 for kernel-Fe, while for kernel-Zn, it ranged from 0.27 (V345-PV-A and V345-PV-B) to 0.88 (HPLET-03-3 and CML319) with a mean of 0.57. Cluster diagram grouped the 20 inbred lines for kernel-Fe into three major clusters (Fig. 2). Cluster A

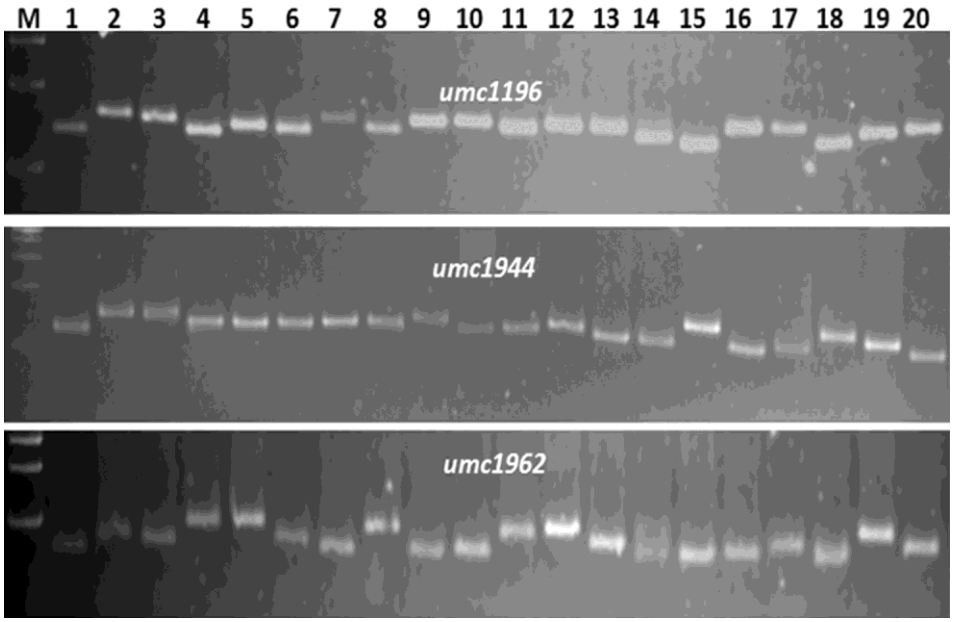

Fig. 1 — SSR polymorphisms among a set of 20 selected inbred lines. M: 100 bp ladder; 1. V335-PV; 2. HKI1105-Q; 3. HKI1128-Q; 4. VQL1-PV; 5. VQL2.-PV; 6. MGUQ-1-opaque; 7. CM501; 8. CML319; 9. HKI323-Q; 10. HKI161; 11.HPLET-03-3; 12.HP-19-01; 13.V345-PV-A; 14.CM150-Q; 15.HKI1105-PV; 16.MGUQ-2-mod; 17.CML336; 18.HKI1105-Q+PV; 19. CM151-Q; 20. V345-PV-B.

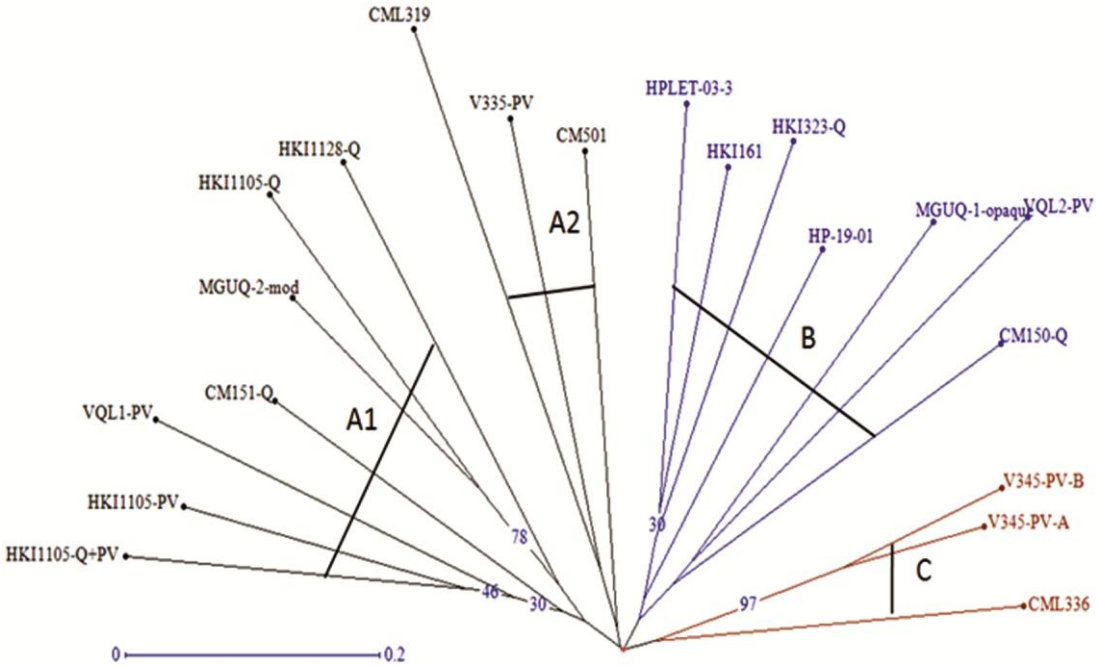

Fig. 2 — Cluster analysis depicting genetic relationship among inbreds for kernel Fe. Bootstrap value of ≥30 is presented.

possesses 10 inbreds which was further divided into two sub-clusters (A1 and A2). Sub-cluster A1 contained seven inbred lines viz., HKI1105-Q+PV, HKI1105-PV, VQL1-PV, CM151-Q, MGUQ-2-mod, HKI1105-Q and HKI1128-Q, whereas sub-cluster A2 consisted of three inbred lines CML319, V335-PV and CM501. Cluster B consisted of seven genotypes *viz.* HPLET-03-3, HKI161, HKI323-Q, HP-19-1, MGUQ-1-opaque, VQL2-PV and CM150-Q, whereas cluster C consisted of three inbred line *viz.* CML336, V345-PV-A and V345-PV-B. In case of kernel-Zn, cluster diagram grouped the 20 genotypes into three

different clusters (Fig. 3). Cluster A contains nine genotypes viz., V345-PV-A, V345-PV-B, CML319, CM151-Q, V335-PV, VQL2-PV, VQL1-PV, HPLET-03-3 and MGUQ-1-opaque. Cluster B consisted of eight inbred lines (HKI161, HKI323-Q, MGUQ-2-mod, HKI1105-Q+PV, HKI1105-Q, HKI1105-PV, CML336 and HKI1128-Q), while cluster C had CM150-Q, CM501 and HP-19-1. Principle coordinate analysis (PCoA) for kernel-Fe depicted diverse nature of the inbreds that were present in the four quadrangles each having five inbreds (Fig. 4). V345-PV-A and V345-PV-B were close to each other in the bottom right quadrangles. HKI1105-PV and HKI1105-Q+PV were also together in the bottom left quadrangle. In case of Zn, PCoA also showed distribution of inbreds across quadrangles (Fig. 5). Eight inbreds were in top left quadrangle, while two were in top right quadrangle. Ten inbreds could be observed in the bottom half, with three and seven inbreds in the left and right quadrangle, respectively. Genetically close inbreds, (i) V345-PV-A and V345-PV-B, and (ii) HKI1105-Q, HKI1105-PV and HKI1105-Q+PV were close to each other.

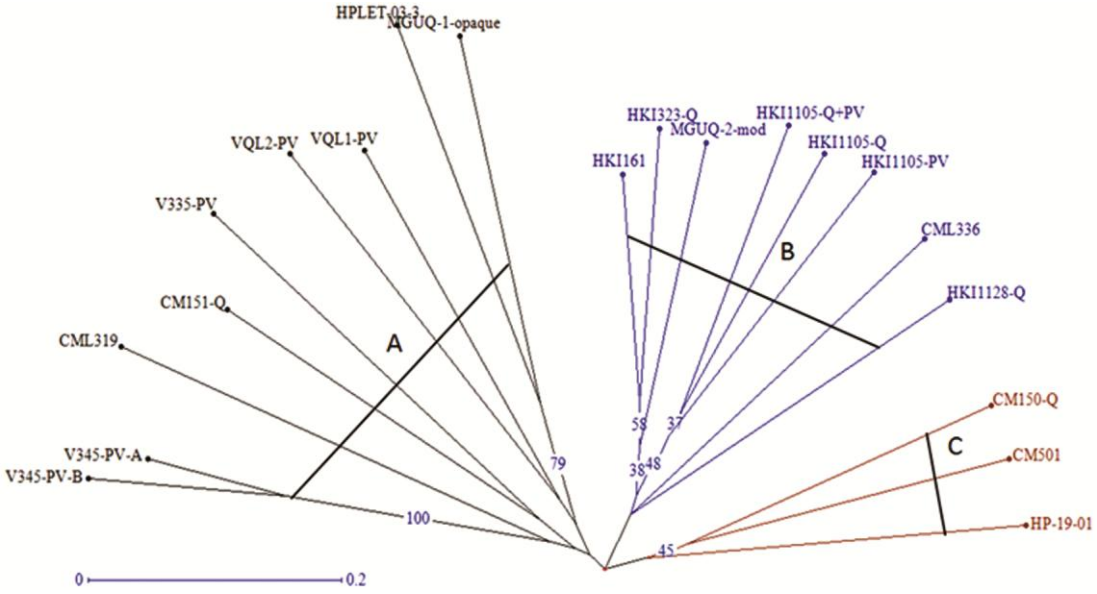

Fig. 3 — Cluster analysis depicting genetic relationship among inbreds for kernel Zn. Bootstrap value of ≥30 is presented.

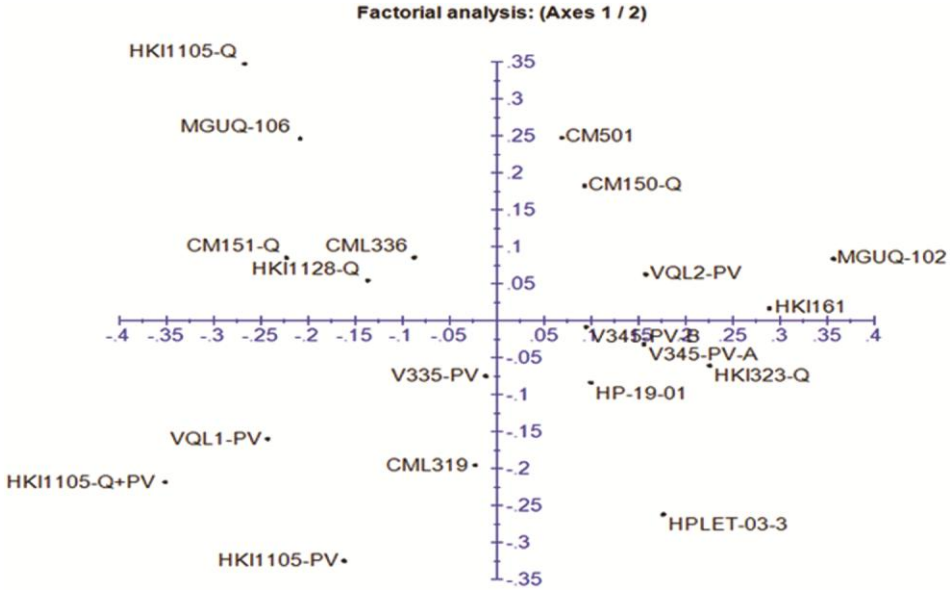

Fig. 4 — Principal coordinate analysis (PCoA) for kernel-Fe in maize inbreds.

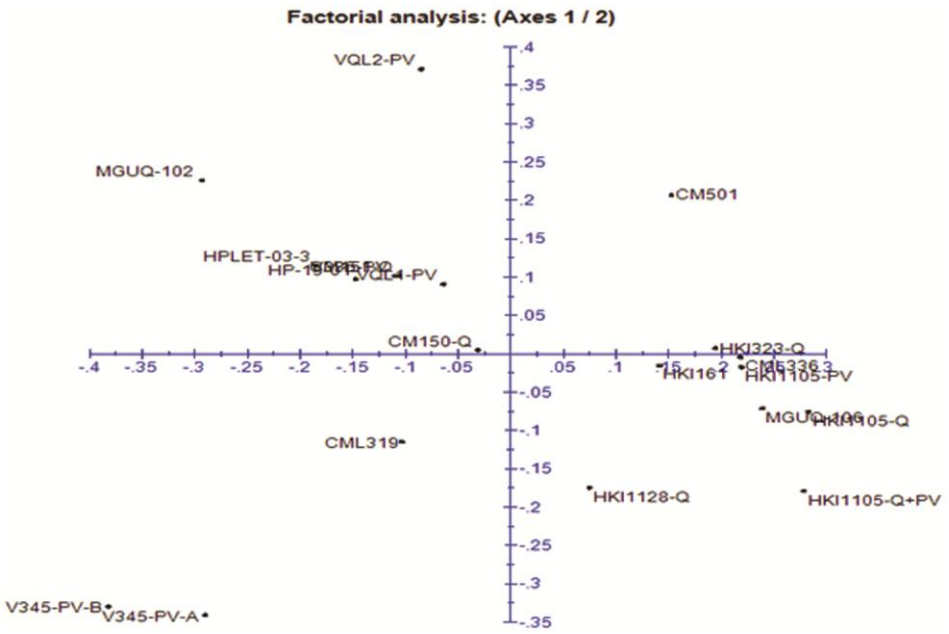

Fig. 5 — Principal coordinate analysis (PCoA) for kernel-Zn in maize inbreds .

Discussion

Genetic Variation for Fe and Zn

Wide genetic variation was observed for kernel Fe and Zn among the inbreds. This suggested that natural genetic variation exists in the germplasm, and genetic improvement of target traits could be undertaken by introgressing the target loci. Sufficient genetic variation for kernel Fe and Zn in maize inbreds has also been reported by Chakraborti *et al*[32] (Fe: 13.23-40.09 mg/kg; Zn: 13.44-46.39 mg/kg) and Mallikarjuna *et al*[33] (Fe: 16.6-83.4, Zn: 16.4-53.2). In the present study, moderate positive correlation between kernel Fe and Zn concentration among the genotypes was observed. This is evident that some of the lines were high in both Fe and Zn. For example, CM501 (Fe: 46.13 mg/kg; Zn: 38.36 mg/kg), V335-PV (Fe: 34.05 mg/kg; Zn: 39.56 mg/kg), MGUQ-1-opaque (Fe: 34.60 mg/kg; Zn: 38.51 mg/kg), VQL1-PV (Fe: 34.54 mg/kg; Zn: 33.88 mg/kg), V345-PV-B (Fe: 36.58 mg/kg; Zn: 33.82 mg/kg), V345-PV-A (Fe: 36.34 mg/kg; Zn: 31.19 mg/kg), and HPLET-03-3 (Fe: 31.00 mg/kg; Zn: 31.22 mg/kg) were identified as promising lines that can be utilized in breeding programme for the improvement of kernel micronutrient concentration. Earlier studies have also reported significant positive correlation between kernel Fe and Zn[34-35]. This could be possibly due to linkage between the genes or pleiotropic effects of the genes governing the accumulation of micronutrients. Positive correlation between kernel Fe and Zn observed in earlier studies is due to co-localization of QTLs for both the traits at the same chromosome, thereby suggesting the feasibility of simultaneous improvement of the both. On the contrary, several researchers reported non association of Fe with Zn. Since, the positive correlation was not of high magnitude, some of the inbreds in the present study possessed contrasting levels of minerals. For example, VQL2-PV was high for Fe (35.40 mg/kg), but was low in Zn (22.98 mg/kg). Similar observations were also recorded for HKI323-Q and CM151-Q.

Molecular Characterization

Four SSR markers *viz*. *nc004*, *umc1556*, *bnlg2336* and *umc1335*, were found to have the selective efficiency in separating one inbred from others by amplification of unique alleles which could be useful in fingerprinting studies. The major allele frequencies observed in the study with low value indicate highly diverse nature of the locus among the selected set of genotypes[36]. The observation of low mean heterozygosity reveals the high level of homozygosity among the inbreds. However, few markers showed some degree of heterozygosity for *bnlg339* (0.16), *umc1125* (0.15) and *dupssr29* (0.11). Various reasons could be attributed to this, of which residual heterozygosity is an important factor, where inbreds tend to segregate for few loci despite repeated cycles of selfing over many generations[37]. Inbreds derived

through doubled haploid technology are fixed for all loci, compared to conventionally bred inbred lines[38]. Other possible reason could be the mutation at specific SSR locus or amplification of similar sequences from different genomic regions due to duplication[39].

Utilization in Breeding Programme

Clustering of all the genotypes under study depicted robust congruence with pedigree data (Table 1). The inbred lines *viz.* HKI1105-Q, HKI1105-PV and HKI-1105-Q+PV developed from HKI1105 through marker assisted selection at IARI, New Delhi, India were grouped together within the cluster A for kernel Fe while, the same were grouped together within cluster B for kernel Zn. Similarly, V345-PV-A and V345-PV-B used as check were also grouped together for both kernel Fe and Zn. Similar pattern of closeness among these lines was also observed PCoA analysis.

All earlier studies have indicated that accumulation of Fe and Zn in maize kernel is governed by many QTLs each having minor effects. Thus, markers with correlation of > 0.30 (with the mean Fe and Zn) were identified. These markers can help accumulating the favourable QTLs to develop new inbreds rich in Fe and Zn. The favourable allele size of the most significant markers associated with high Fe (*umc1464*: 130 bp, *umc1016*: 170 bp, *bnlg1884*: 250 bp, *mmc0171*: 180 bp, *umc1230*: 230 bp, *umc1518*: 160 bp and *bnlg1056*: 110 bp) and high Zn (*bnlg1732*: 110 bp, *umc1556*: 210 bp, *umc1019*: 100 bp, *umc1015:* 140 bp, *bnlg2336:* 110 bp, *umc1335*: 150 bp, *umc1386*: 230 bp, *umc1335*: 200 bp and *bnlg1740*: 180 bp) can be effectively used in the breeding programme. CM501, VQL2-PV, HKI1105-PV and V345-PV-A/ V345-PV-B are promising genotype for Fe and genetically distant as they belong to different clusters. This suggests that these inbreds possess favourable but diverse QTLs linked to kernel-Fe. The possible heterotic combinations viz., CM501 × VQL2-PV, CM501 × HKI1105-PV, CM501 × V345-PV-A/ V345-PV-B, VQL2-PV × HKI1105-PV, VQL2-PV × V345-PV-A/ V345-PV-B and HKI1105-PV × V345-PV-A/ V345-PV-B, can therefore be attempted to develop high Fe maize hybrids. In case of Zn, CM501, V335-PV, VQL1-PV, MGUQ-1-opaque and V345-PV-B were genetically distant promising lines. Several combinations viz., CM501 × V345-PV-B, CM501 × V335-PV, CM501 × VQL1-PV, CM501 × MGUQ-2-mod, V345-PV-B × VQL1-PV, V345-PV-B × MGUQ-2-mod, V335-PV × VQL1-PV and V335-PV × MGUQ-2-mod can be made to develop heterotic hybrids with high kernel-Zn. Among the combinations, CM501 × V345-PV-B can be attempted to develop high yielding hybrid rich in both Fe and Zn. It is important to mention here that the grouping has been based on the SSRs linked to QTLs for Fe and Zn. Several researchers have used genome diversity using genome wide SSRs to predict heterotic grouping in maize. Since, SSRs linked to QTLs for Fe and Zn are distributed throughout the genome, they effectively provide information of genome diversity as well. Further, CM501, VQL2-PV HKI1105-PV and V345-PV-A/ V345-PV-B for Fe, and CM501, V335-PV, VQL1-PV, MGUQ-1-opaque and V345-PV-B for Zn can be combined separately to develop populations from where inbreds with accumulated QTLs for Fe and Zn can be identified.

On the other hand, high Fe but low Zn lines (VQL2-PV, HKI323-Q and CM151-Q) can be crossed with high Zn but low Fe lines (V335-PV and MGUQ-1-opaque), to select F_2 segregants high in both Fe and Zn. These inbreds with favourable QTLs linked to Fe or Zn would serve as rich genetic resource. Inbreds with high Fe and Zn are limited in nature, and accumulation of favourable loci using marker-based information will help developing suitable donor lines that can be effectively used in the breeding programme. Pandey *et al*[40] characterized a set of QPM inbreds using SSRs linked to QTLs for endosperm modifiers and amino acid accumulation in maize, and based on the clustering pattern and phenotypic performance, a set of cross combinations were identified to develop QPM inbreds with enhanced kernel modifications and quality. In the present study, SSRs linked to Fe and Zn were used to genetically characterize a set of QPM and provitamin-A rich maize inbreds. The information generated here assumes great significance in the maize biofortification programme. This is the first report of molecular analyses of maize inbreds using SSRs linked to QTLs for Fe and Zn. In conclusion, maize inbreds studied here varied significantly for kernel Fe and Zn, and promising inbreds for either and both the traits inbreds were highly homozygous, and unique alleles were identified in specific inbreds. Cluster diagram and PCoA depicted the diverse nature of the inbreds. Specific cross combinations were identified to mobilize favourable and diverse QTLs for the development of new inbreds with enhanced Fe and Zn. The information generated here can be effectively utilized in the maize biofortification programme.

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