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Assessment of genetic diversity in quality protein maize(QPM) inbreds using ISSR markers

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Abstract

Genetic diversity of 49 maize inbreds was assessed using twelve ISSR primers. A wide variation in PCR products was revealed in terms of size (280 to 3000bp), extent of polymorphism (94.87%) and number of bands (4-9). As a whole, 78 ISSR bands were produced (including four monomorphic bands) with an average of 6.5 bands per primer and the maximum number of bands (9) being produced by primer OUAT-8. Five ISSR primers (OUAT-8, OUAT-9, OUAT-15, OUAT 17 and OUAT-18) revealed higher PIC value (around 0.70) along with 100% polymorphism indicating better allelic diversity. While, ISSR primer OUAT-15 revealed higher number of polymorphic bands (8) with 100% polymorphism as well as considerably high PIC and Rp values. Thus, such an informative and discriminative primer is of immense value for the study of genetic diversity in a set of maize genotypes. The similarity index values ranged from 0.3 to 0.9 with an average of 0.522 and BQPM-1-14 maintained the highest genetic distance as revealed from its lowest average similarity coefficient value (0.393) with rest of the genotypes. BQPM 1-14, BQPM 1-8, BQPM 3-10, BQPM 6-8 and B 1110-7-2 were identified to be highly divergent among the test inbreds which could be sorted out as valuable materials for heterosis breeding for production of single cross hybrids.

Key words

Genetic diversity, Quality protein maize inbreds, ISSR markers

Introduction

Maize (*Zea mays* L.2n =20) has worldwide significance as human food, animal feed and a source of large number of industrial products. It is the fourth most important cereal crop in India next to rice, wheat and sorghum. The area, production and productivity in India were 4.5 m. ha, 4.3 m.t. and 9.56 q ha^{-1}, respectively in 1961 which increased to 6.2 m. ha, 12.7 m.t and 20.48 q ha^{-1} in the years 2005-06. This made India rank fourth in the world. But still, to address global demands due to continuing and alarming population growth and malnutrition; genetic improvement of productivity and nutritional quality in this crop is unequivocal (Tester and Langridge, 2010). The normal maize grain harbours 9.0 % crude protein and is deficient in lysine and tryptophan. In contrast, the quality protein maize endosperm harbour sufficiently high seed protein bound lysine (>30%) and tryptophan (>55%) owing to the presence of *opaque 2* mutation. Therefore, in recent years, a major emphasis has been laid on conversion of normal maize genotypes to opaque-2 versions. Understanding genetic diversity and the knowledge of genotypic relationship among inbreds is useful for production of high yielding hybrids (Bauer *et.al.,* 2007).

Assessment of genetic diversity at expression level such as those based on agro-economic traits (morphological), protein and isozyme profiling are influenced by environment, particularly at developmental stages. Besides, these are limited in number and many often hinder recovery of reproducible polymorphism. On the contrary, molecular markers are more powerful and reliable in the study of genetic diversity (Schlotter, 2004; Schulman, 2007) owing to its plasticity, ubiquity and stability over environments (Bernardo, 2008). DNA based markers are capable of producing abundant number of polymorphic loci from all plant tissues regardless of growth, differentiation, development or status of cells. Among different DNA markers, ISSR markers offer added advantage of both RAPD and SSR marker systems. The

ISSR-PCR technique generates highly polymorphic multi-locus dominant markers (Reddy *et al.,* 2002; Oliveira *et al*., 2010). The technique is considered simple and fast like RAPD, but has more stringency than RAPD. The technique is *in vogue* and is used for the study of genetic diversity, phylogeny, genetic coding, genomic mapping and evolutionary biology (Reddy *et al*., 2002) in many crops. A set of 49 promising inbreds were purposefully selected a from large number of quality protein maize (QPM) inbreds based on vigour, yield potential and quality attributes. In the present investigation, an attempt was made for detailed study of genetic diversity of these inbreds, following ISSR analysis to further sort out a few elite highly divergent inbred lines for production of QPM hybrids through single crosses.

Materials and Methods

Isolation of DNA : Genomic DNA of each of the 49 QPM inbreds (Table 1) was isolated from two gram tender young leaves of aseptically grown seedlings on the same day of collection using standard sodium dodecyl sulphate method (Dellaporta *et al.,* 1983) with minor modification. The plant materials were homogenized in liquid nitrogen and extracted with extraction buffer(100mM Tris-HCl pH 8.0, 20mM EDTA, 0.5M NaCl, 7M Urea, 0.1% β-ME and 2% SDS) at 65 ºC for 1 hr with occasional shaking and an equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added and centrifuged at 12000rpm for 15 min at 4 ºC. The supernatant was added with equal amount of ice cold absolute ethanol and kept overnight to precipitate DNA. The intact genomic DNA was hooked out and washed with 70% ethanol 2-3 times and finally re-dissolved in TE buffer (10mM Tris-HCl, pH-8.0 and 1mM EDTA).

DNA was purified by DNase free RNase-A (GeNei) @ 20µg per ml of DNA extract to remove contaminating RNAs. Finally, DNA was quantified through UV-VIS Nanodrop-2000 spectrophotometer (Thermo Electron Scientific Instruments LLC, USA) at 260nm and the quality of DNA was checked using the ratio of absorbance at 260nm and 280nm. Each sample of DNA was diluted to a working concentration of 10ng μl^{-1} for PCR analysis.

ISSR analysis : Genomic DNA sample of each maize inbred was individually primed and amplified using twelve ISSR primers (Chromos Biotech. Ltd., India). PCR amplification was performed in a reaction volume of 25µl containing 1X reaction buffer (10mM Tris HCl, pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin), 2.5mM each of dNTPs, 10ng of single random primer, 20ng of genomic DNA and 1 unit of Taq polymerase (Genei, Bangalore).

Table 1 : List of inbred lines tested

Sl.No.	Genotype	Pedigree	Sl.No.	Genotype	Pedigree
01.	BQPM -3-7	DMR Population-3	26.	BQPM-5-20	DMR Population-5
02.	BQPM-5-2	DMR Population-5	27.	BQPM-6-4	DMR Population-6
03.	BQPM-7-4	DMR Population-7	28.	BQPM-7-8	DMR Population-7
04.	BQPM-9-2	DMR Population-9	29.	BQPM-7-14	DMR Population-7
05.	BQPM-9-16	DMR Population-9	30.	BQPM-8-1	DMR Population-8
06.	BQPM-10-9	DMR Population-10	31.	BQPM-8-5	DMR Population-8
07.	BQPM-1-15	DMR Population-1	32.	BQPM-8-7	DMR Population-8
08.	B1110-7-2	DMR Population-7	33.	BQPM-8-11	DMR Population-8
09.	BQPM-3-10	DMR Population-3	34.	BQPM-8-12	DMR Population-8
10.	BQPM-6-8	DMR Population-6	35.	BQPM-8-15	DMR Population-8
11.	BQPM-1-8	DMR Population-1	36.	BQPM-9-4	DMR Population-9
12.	BQPM-1-14	DMR Population-1	37.	BQPM-9-19	DMR Population-9
13.	B1130-7	DMR Population-7	38.	BQPM-10-1	DMR Population-10
14.	B1131-8	DMR Population-8	39.	BQPM-10-4	DMR Population-10
15.	BQPM-1-2	DMR Population-1	40.	BQPM-10-13	DMR Population-10
16.	BQPM-1-3	DMR Population-1	41.	HKI-164-4(1-3)-2	HKI population-164
17.	BQPM-1-6	DMR Population-1	42.	HKI-164-7-6*161-2	HKI population-161
18.	BQPM-1-7	DMR Population-1	43.	HKI-191-1-2-5	HKI population-191
19.	BQPM-2-4	DMR Population-2	44.	HKI-193-1	HKI population-193
20.	BQPM-2-10	DMR Population-2	45.	HKI-193-2	HKI population-193
21.	BQPM-2-15	DMR Population-2	46.	CLQ RCY Q 40	CIMMYT Popn.-40
22.	BQPM-2-18	DMR Population-2	47.	BQPM-3-124	DMR Popn.-3
23.	BQPM-3-4	DMR Population-3	48.	CML-163-13	CIMMYT Popn-163
24.	BQPM-5-9	DMR Population-5	49.	CML-195	CIMMYT Popn-195
25.	BQPM-5-19	DMR Population-5			

Source : Sl. 1 to 40 and Sl.47 received from DMR, New Delhi, India, Sl. 41-45 from RS, Kernal, Haryana, India and Sl. 46, 48 and 49 from CIMMYT, Hyderabad, INDIA

Table 2 : Amplified products with different ISSR anchored primers in 49 advanced inbreed lines of maize

Sl.No.	Primer Code	Sequences (5'-3')	GC content (%)	*Tm (ºC)	*Tan (ºC)	Poly-morphic bands	No. of mono-morphic bands	Total bands	% poly-mor-phism	PIC value	Resolving power (Rp)	Range of fragme-ntsize (bp)
1	OUAT-1	(AACC)6T	48.0	63.1	56.0	6	1	7	85.7	0.595	8.408	700-3000
2.	OUAT-3	(TGGT)6C	52.0	64.6	56.0	5	0	5	100.0	0.349	8.000	550-1900
3.	OUAT-4	(CCAA)6C	52.0	64.6	61.0	7	1	8	87.5	0.620	9.265	710-1750
4.	OUAT-6	(AACT)6T	24.0	53.1	56.0	4	0	4	100.0	0.297	6.530	720-1480
5.	OUAT -8	CGA(CTC)4	66.7	53.3	66.7	9	0	9	100.0	0.643	8.367	320-2950
6.	OUAT- 9	(CT)8T	47.1	50.4	40.0	7	0	7	100.0	0.720	5.959	280-1500
7.	OUAT-10	(TC)8T	47.1	50.4	47.1	5	1	6	83.3	0.577	7.387	340-1420
8.	OUAT-12	(AG)8C	52.9	52.8	46.6	6	0	6	100.0	0.463	8.653	600-2000
9.	OUAT 14	(AGC)5CA	64.7	57.6	60.0	5	1	6	83.3	0.501	8.204	970-2020
10.	OUAT-15	(GGC)5AT	88.2	67.2	58.0	8	0	8	100.0	0.630	9.632	300-1620
11.	OUAT-17	(GGC)5TA	88.2	67.2	56.0	6	0	6	100.0	0.651	7.020	740-1950
12.	OUAT-18	(AGC)5CG	70.6	60.0	61.0	6	0	6	100.0	0.733	6.163	730-1690
		Total				74	4	78				

Note: Range of amplicon size as a whole for ISSR marked bold. *-"Tm" and "Tan" denote melting and annealing temperature of ISSR primers.

DNA amplification was carried out in the Gene Pro Thermocycler (Bioer Tech. Co., Ltd, Japan), programmed for 5 min at 94 ºC, 40 cycles of 1 min at 94 ºC, 1 min at specific annealing temperature (Table 2) and 2 min at 72 ºC and final extension for 4 min at 72ºC followed by storing at 4 ºC till loading to agarose gel. The amplified products were loaded in 2.0% agarose gel containing 0.5 mg ml^{-1} of ethidium bromide and electrophoresed at a constant voltage (50V). PCR conditions were optimized to yield reproducible result. Only reproducible amplified fragments (about 0.5-mm apart) were scored. The gels were documented by gel doc system (Fire Reader-Uvtec, Cambridge, UK) for scoring the bands. The size of amplicons was determined by comparing with lambda DNA ladder (500bp) with known size (bp) fragments. The presence and absence of bands were scored as 1 and 0 respectively and each band, corresponding to each amplified fragment was treated as a unit character (as these are dominant markers) to determine variation among genotypes. Polymorphism information content (PIC) was calculated based on the number of bands per primer, using the formula PIC = $\Sigma (1 - Pi^2)/n$, where Pi is the frequency of the i^{th} band amplified by the primer and n= total no. of bands produced by the primer. Whereas, the resolving power (Rp) of a primer was estimated as Rp=Σ Ib, where Ib (band informativeness) =1–[2×(0.5–pi)] and pi is the proportion of inbreds showing i^{th} band (Prevost and Wilkinson, 1999).

The binary data matrix of ISSR score was analysed following NTSYS software programme to estimate Jaccard's similarity co-efficient (Jaccard, 1908) values. Dendrogram was constructed using unweighted paired group method with arithmetic averages (UPGMA)-phenograms (Sokal and Michener, 1958) employing sequential agglomerative hierarchic and non-overlapping Clustering (SAHN).

Results and Discussion

In quality protein maize, the genetic base is narrow as it is originated only from the flint /semi-dent type of common maize with high lysine and tryptophan content. Therefore, critical characterization and evaluation of inbreds are indeed essential and better knowledge about them will allow detection of possible genotypes to be used in production of QPM hybrids (Gepts, 2006; Sudre *et al.,* 2010).

ISSR have been standardized and employed successfully by different workers (Teixeira *et al.,* 2011; Carvalho *et al.,* 2002) to analyse samples of maize. The success in generating wide range of polymorphic loci depends on proper choice of primers for DNA amplification. The optimum number of primers required to differentiate two or more genotypes may vary with the test materials used. When variation in genotypes is high, the use of few primers can serve the purpose to generate useful information. Dhanaraj *et al*. (2002) used only seven primers to distinguish among cultivars. However, it is *in vogue* to use more number of primers to differentiate closely related genotypes in maize. In the present investigation, initially twenty five ISSR primers were examined out of which six ISSR primers gave smeared background without distinct fragments and a few of ISSR primers did not produce any amplified products of DNA. As a result, twelve ISSR primers were used for genomic DNA amplification of 49 inbreds of maize (Table 2). In the present investigation, ISSR revealed a wide array of PCR products with a range of 280bp to 3000bp and produced 4-9 bands with an average of 6.5 bands per primer and the maximum number of bands (9) being produced by primer OUAT-8 (Fig 1). As a whole, ISSR produced 74 polymorphic amplicons out of total 78 scorable amplified products and thus resulted in tremendous high level of

polymorphism (94.87%) in the present set of material. Carvalho *et al.* (2002), reported that 75.8% polymorphism with ISSR markers in maize. Total number of bands scored varied widely among the present set of genotypes. It ranged from 37 bands in BQPM-3-124 to as high as 57 bands in BQPM-9-2. The binary data matrix of the presence and absence of bands resolved 2,097 polymorphic amplicons out of total 2,293 PCR products resulting in 91.45% polymorphism across the test genotypes using 12 ISSR primers. High level of polymorphism obtained in the present investigation may be ascribed to higher genetic variation in the developed inbreds.

Primer length, GC content, melting temperature (Tm) and annealing temperature are considered as critical factors for amplification of DNA. Larger motif ISSR primers that would cause dimerism are usually avoided. GC-rich primers are expected to yield more polymorphism in agarose gel. In the present pursuit, two ISSR primers OUAT-8 (GC= 66.7%) and OUAT-15 (GC=88.2%) produced more number of amplicons as well as higher level of polymorphism (Table 2). Blair *et al.* (1999) observed that tetra-nucleotide motif ISSR primers with GC-content more than 50% amplified an average of 14.7 bands, while those that were AT-rich (75% or greater AT content) produced no bands at all over a wide range of annealing temperature. Since annealing temperature is primer specific, it was determined for each primer over a possible range of temperature to visualize maximum number of bands with good resolution. Sometimes ISSR primers are designed with 1-3 anchored nucleotides at either 3' or 5' end. The anchor allows only a subset of the targeted inter-repeat regions to be amplified. In the present investigation, a poly-CTC primer OUAT-8 with anchor CGA at 5' end produced 100% polymorphism (Table 2, Fig 1). Similarly, ISSR primers OUAT 9 and OUAT 12 with anchor T and C respectively at 3' end also revealed 100% polymorphism (Table 2, Fig 2 & Fig 3). In the present investigation, ISSR primers e.g., OUAT-1, OUAT-4, OUAT-10 and OUAT-14 produced one monomorphic band each (Table 2) having amplicon size 1250bp, 1140bp, 480bp and 2020bp respectively. However, band, 1500bp, amplified by OUAT-9 was specific to inbread BQPM 2-15 and BQPM 8-15, while the amplicon 1300bp produced by the same primer (OUAT-9) was unique to BQPM 7-14 and BQPM 9-4. Since ISSR markers was dominant in nature, the absence of a specific band could also serve as valuable information for varietal discrimination and assessment of genetic relationship. In this context, both 1320bp and 450bp, produced by OUAT 8, were specifically absent in BQPM 1-2. The absence of characteristic bands in few inbred might be attributed to the fact that these markers could be linked with negative traits (Warburton *et al*., 2002) identified 53 genotype specific SSR markers for varietal discrimination of a set of seven tropical maize populations and 57 inbred lines at CIMMYT.

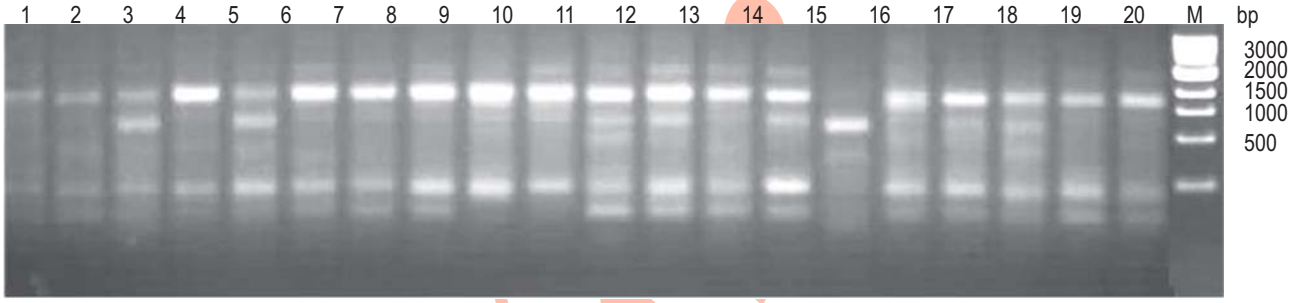

Fig. 1 : ISSR profile of different maize inbreds amplified with primer OUAT -8. M=DNA molecular marker, Lane 1-20 : BQPM-3-7, BQPM-5-2, BQPM-7-4, BQPM-9-2, BQPM-9-16, BQPM-10-9, BQPM-1-15, B1110-7-2, BQPM-3-10, BQPM-6-8, BQPM-1-8, BQPM-1-14, B1130-7, B1131-8, BQPM-1-2, BQPM-1-3, BQPM-1-6, BQPM-1-7, BQPM-2-4 and BQPM-2-10

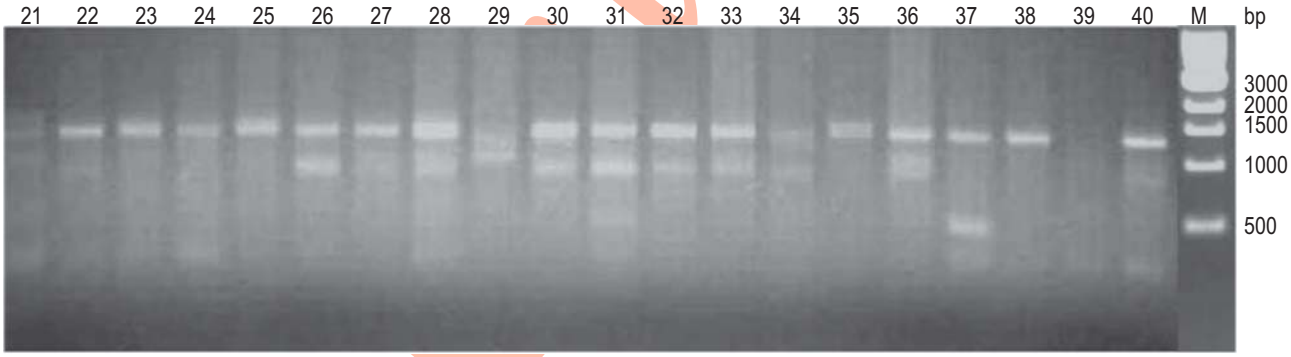

Fig. 2 : ISSR profile of different maize inbreds amplified with primer OUAT -9. M=DNA molecular marker, Lane 21-40 : BQPM-2-15, BQPM-2-18, BQPM-3-4, BQPM-5-9, BQPM-5-19, BQPM-5-20, BQPM-6-4, BQPM-7-8, BQPM-7-14, BQPM-8-1, BQPM-8-5, BQPM-8-7, BQPM-8-11, BQPM-8-12, BQPM-8-15, BQPM-9-4, BQPM-9-19, BQPM-10-1, BQPM-10-4 and BQPM-10-13

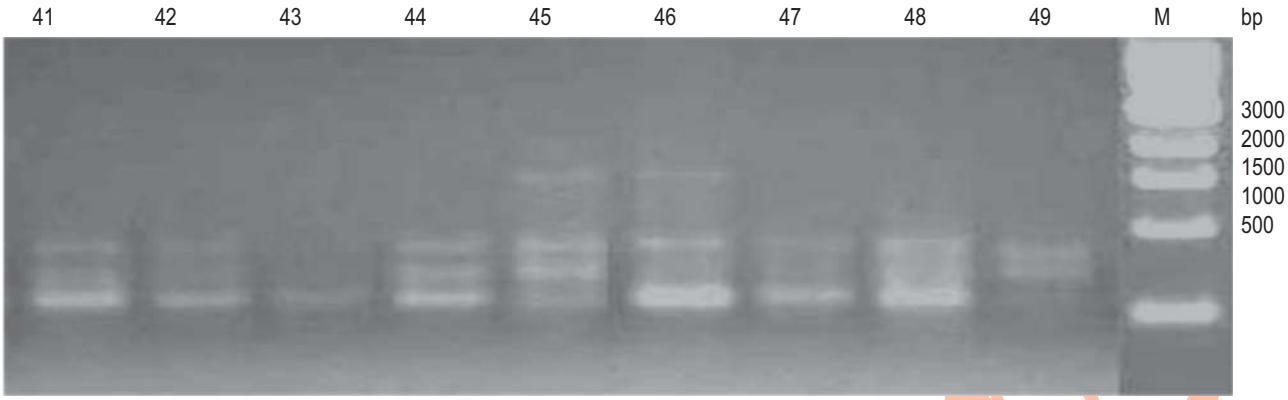

Fig. 3 :. ISSR profile of different maize inbreds amplified with primer OUAT -9. M=DNA molecular marker, Lane 21-40 : BQPM-2-15, BQPM-2-18, BQPM-3-4, BQPM-5-9, BQPM-5-19, BQPM-5-20, BQPM-6-4, BQPM-7-8, BQPM-7-14, BQPM-8-1, BQPM-8-5, BQPM-8-7, BQPM-8-11, BQPM-8-12, BQPM-8-15, BQPM-9-4, BQPM-9-19, BQPM-10-1, BQPM-10-4 and BQPM-10-13

Similarly, Kassahun and Prasanna (2002) identified 36 SSR loci to discriminate 23 QPM lines.

DNA banding pattern of each of the genotypes is expected to differ if they are genetically different. Even subtle difference at genotypic level which other-wise some time could not be possible to differentiate by phenotyping, can be confirmed by use of markers. DNA markers, using of most informative primers, could pave the way for success. Polymorphism information content (PIC)- a measure of allelic diversity produced by a primer was estimated for all ISSR primers. The values ranged from 0.297(OUAT-6) to 0.733 (OUAT-18). Eight out of 12 ISSR primers were higher than the PIC mean value (0.564). Further, it is worth noting note that PIC values of five ISSR primers (OUAT-8, OUAT-9, OUAT-15, OUAT-17 and OUAT-18) were around 0.70 along with 100% polymorphism.

The ability of primers to distinguish between inbred genotypes was assessed by calculating their resolving power (Rp). Rp-value for different primers ranged from 5.959 in OUAT-9 to as high as 9.632 in OUAT-15 with a mean value of 7.799. It is worth noting that ISSR primer OUAT-15 revealed higher number of polymorphic bands (8) with 100% polymorphism as well as considerably high PIC and Rp values. Thus, such informative and discriminative primer is of immense value for study of genetic diversity in a set of maize genotypes.

Molecular markers allow direct comparison of similarity of genotypes at DNA level using large number of polymorphic loci in a set of genotypes could reveal heterotic/ divergent groups and as such avoiding of the increasing uniformity in the elite germplasm could ensure long-term selection gains (Prasanna and Hoisington, 2003). Besides, similarity coefficient value between each pair of genotypes is likely to give a clear picture of the extent of genomic homology in terms of gene content and nucleotide sequence (Mohammadi *et al., 2002).* Binary data scored in terms of presence or absence of band for each of the test genotypes were used to estimate similarity index. Similarity index value in each pair of genotypes ranged from 0.3 to as high as 0.9 between BQPM 8-1 with BQPM 8-7, with an average of 0.522 which indicated wide genetic diversity among the test genotypes.

Out of total 1176 combinations for pairs of inbreds, similarity coefficient value of 0.3 was revealed only in 3.31%, 0.4 to 0.5 in 80.44% combinations, followed by 0.6 to 0.9 in 16.24% of the combinations respectively. Based on ISSR markers, the most divergent genotype exhibiting highest genetic distance with rest of the genotypes was identified to be BQPM-1-14 as it showed lowest similarity coefficient value with rest of the genotypes (average SI value= 0.393). Besides, inbreds HKI-164-4(1-3)-2 and BQPM-3-10 also showed high level of average genetic distance (average SI value 0.412 and 0.414 respectively) with other inbreds.

Genetic diversity can be studied based on morphological, biochemical and molecular level. However, molecular markers have special advantages over others as they show detail genetic differences in a faster way and without any environmental influence (Saker *et al.,* 2005; Souza *et al.,* 2008). Therefore, in recent years, considerable emphasis has been made on the development of genetically uniform inbred lines (Troyer, 2001), and making selection decisions using DNA markers to generate successful heterotic hybrid in all major crops.

The whole range of 12 ISSR primer-based DNA profiles comprising 78 distinct scorable bands in 49 test inbreds revealed eight genetic clusters at 52% phenon level (Fig 4). Among the test genotypes, BQPM 1-14 and BQPM 1-8 and BQPM 3-10 were sequentially separated from rest of the inbreds and constituted Cluster-I and Cluster II, Cluster III respectively. Cluster IV included two inbreds e.g., BQPM 6-8 and B1110-7-2 while, Cluster V comprised a single inbred HKI-191-1-2-5. Cluster VI was a multi-inbred cluster which included eight inbreds e.g., BQPM 3-12, BQPM 10-1, BQPM 8-1. BQPM 5-9, BQPM 1-2, BQPM 1131-8, BQPM 1130-7 and BQPM 7-4. The rest of the genotypes were distributed into two multi-inbred clusters e.g.,

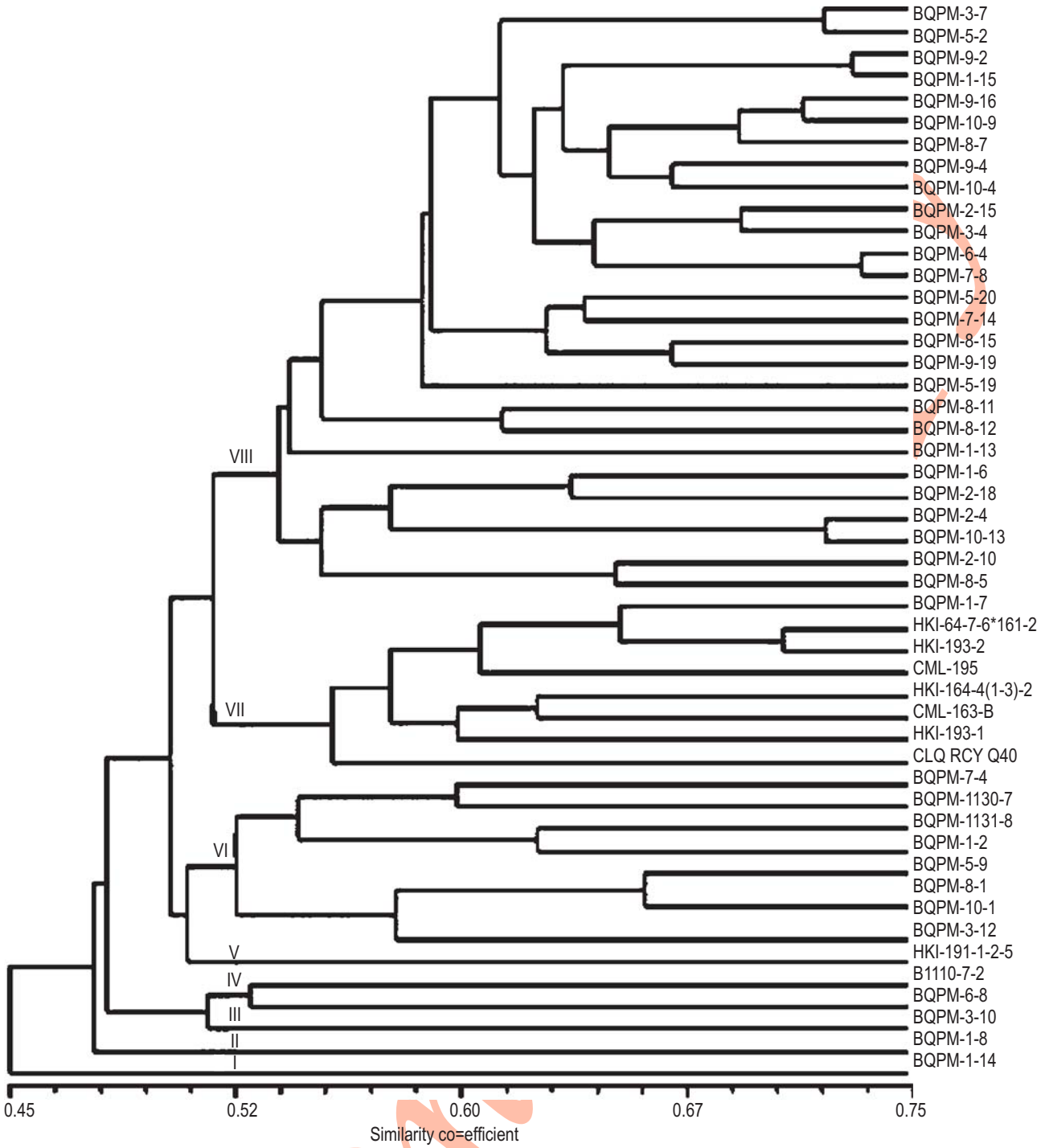

Fig. 4 : Dendrogram showing genetic diversity in a set of 49 maize inbreds based on ISSR markers

Cluster VII and Cluster VIII which grouped 8 and 27 inbreds. Kassahun and Prasanna (2002) analysed 13 Indian QPM inbreds developed through the National programme at IARI and 10 QPM lines from CIMMYT, Mexico using SSR markers. The cluster analysis distinguished the Indian QPM inbreds from those developed at CIMMYT.

The clustering of genotypes using two dimensional and three dimensional scaling based on PCA values (Fig. 5a, b) was found to be more or less consistent with that of UPGMA analysis. It is worth noting that V12 (BQPM 1-14), V11 (BQPM 1-8), V9 (BQPM 3-10), V10 (BQPM 6-8) and V8 (B 1110-7-2) which were initially separated from rest of the test genotypes in case of UPGMA

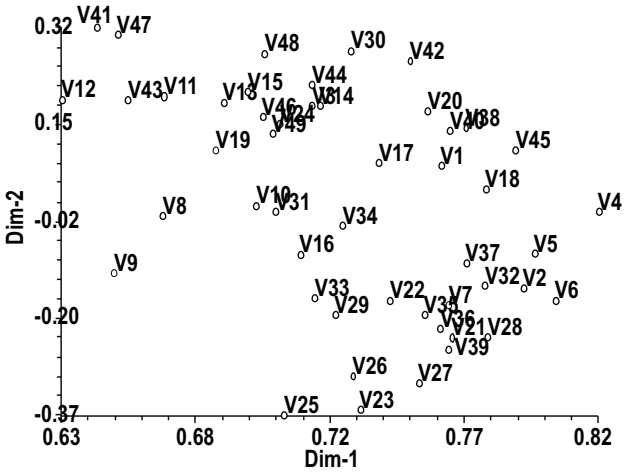

Fig. 5a : 2-dimensional scaling (without vectors) of principal co-ordinates 1 and 2 using ISSR markers in 49 inbreds of maize

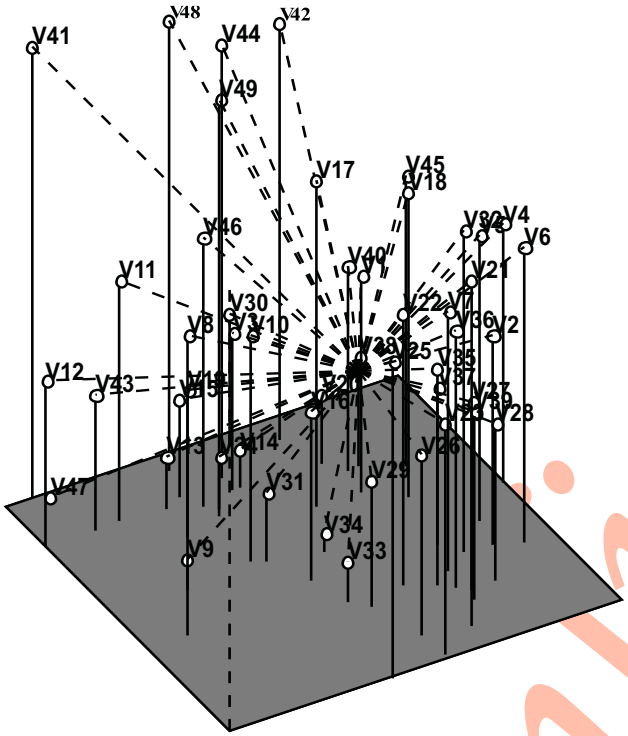

Fig. 5b : Three dimensional scaling (with vectors) of principal coordinates 1, 2 and 3 using ISSR markers

clustering (Fig. 4) were also seen to be sorted out to diverse extreme positions, based on PCA values. Thus, these inbreds included in Cluster-I, II, III and IV maintained wide genetic divergence with rest of the genotypes and hence these could be sorted out as valuable materials for heterosis breeding for production of single cross hybrids. Thus, ISSR markers are helpful in maize breeding, particularly for assignment of lines to heterotic groups and selection of appropriate parental lines for hybrid combination.

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