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SHORT COMMUNICATION

MOLECULAR ANALYSIS OF MAIZE HYBRIDS AND THEIR PARENTS USING ISSR AND PROTEIN PROFILING

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PCR-based Inter Simple Sequence Repeat (ISSR) markers and protein profiling on SDS-PAGE were employed to assess genetic diversity in four maize hybrids and their parents. The DNA was isolated from CTAB extraction method. In ISSR analysis 3 primers gave amplified products. They gave 35 scorable fragments with 100 percent polymorphism and gave 11.67 polymorphic bands per primer. ISSR profiling similarity matrix was obtained using Jaccard Similarity Coefficient was observed up to 0.75 and on this basis a dendrogram was constructed with UPGMA method. The soluble protein was extracted from maize seeds using 0.2 M phosphate buffer (pH 7.4). The concentration of soluble proteins ranged between 20.0 to 90.0 mg/g of seeds. For protein profiling 10% and 15% polyacrylamide gels were used to resolve polypeptides through SDS-PAGE. 24 scorable bands were resolved on 10% gel of which 22 bands were polymorphic (91.67%) and 19 scorable bands were resolved on 15% gel in which 17 bands were polymorphic (89.47%). In protein profiling Jaccard Similarity Coefficient lie between 0.21 to 0.65. According to the dendrogram of ISSR and protein profiling, hybrids resembled more with their female parent.

Keywords: **Genetic relationship, ISSR, maize, polymorphism, protein profiling**

Knowledge about the degree and distribution of genetic diversity and relationship among breeding material has a significant effect on any crop improvement programme. Significant progress has been made in relation to maize improvement in India using traditional breeding strategies (Dhillon and Prasanna 2001), considerable scope exists to further enhance maize productivity. Morphological markers are complex and were used for the individual identification and diversity studies, they may be affected by environmental effects and cultivation practices. In contrast to the morphological markers molecular markers are now available (Khan *et al.* 2000) in plant system, which improves the efficiency of conventional plant breeding. Molecular studies increasingly play an important role in crop improvement programme. Genetic variability at the molecular level in plants has been analyzed by various techniques. The advantage of the Polymerase Chain Reaction favored the development of different molecular techniques such as Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR or micro-satellite), Sequence Tagged Sites (STS), Random Amplified Micro-satellite Polymorphism (RAMP) and Inter-Simple Sequence Repeat Polymorphic DNA (ISSR).

Seed storage proteins, the electrophoretic markers appear to be due to neutral genes, which are not linked to any loci that affect the cultivar morphology and

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physiology. They are rapid, relatively cheap, eliminate the need to grow plants to maturity and are largely unaffected by the growth environment. Seed storage proteins profiles are still powerful tools for determining genetic homology at the molecular level and for solving problems in systematic methodology (Ladizinsky and Hymowitz 1979).

The biochemical methods in evaluation of genetic variability, have some disadvantages such as that they are profoundly influenced by tissue specificity and developmental stage. This disadvantage can be overcome by using the electrophoretic markers of conservative proteins like seed storage proteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used, due to its reliability and simplicity in describing the genetic structure of crop germplasm (Gepts *et al.* 1989).

Numerous seed protein profile studies have been done with various plant species, such as rice (Gramineae) (Aliaga-Morel *et al.* 1987) *Capsicum sp.* (Solanaceae), (Panda *et al.* 1986) *Ricinus communis* (Euphorbiaceae) (Sathaiah and Reddy 1985) *Manihot sp*. (Euphorbiaceae), (Grattapaglia *et al*. 1987) and *Arachis* sp. (Leguminosae) (Bianchi-Hall *et al.* 1993, Lanham *et al*. 1994). In this study we used these techniques to know the genetic differences and similarities between different maize hybrids and their parents.

A set of four maize hybrids and their parents were used for present investigation. The details of genotypes is given in Table 1. Total DNA was extracted from bulked leaves germinated on filter paper in the dark at ambient temperature. After a week the etiolated leaves were harvested grounded in liquid nitrogen and the DNA was extracted using CTAB extraction procedure (Doyle and Doyle 1987). RNA was removed by treating the sample with 0.5µl RNase A per 100 µl of DNA solution at 37°C for one hour. DNA sample were quantified in UV spectrophotometer. The quality of isolated DNA was judged on agarose gel electrophoresis (0.8%) using lambda uncut marker as a standard.

Table 1. Brief description of different maize genotypes and their parents

S.No.	Genotypes	Code No.	Crop duration (days)	Yield (kg/ha)	Seed type
1*	EI - 460	G1	81-85	2435.00	White, bold
2	EI - 472	G2	83-86	2335.00	White, bold
3	EH -1389	G3	80-83	3878.90	White, bold
4	EI - 628	G4	90-95	3075.00	White, medium size
5	EI - 563	G5	85-86	4460.00	White, bold
6	EH -1491	G6	84-87	4363.75	White, bold
7*	EI - 460	G7	81-85	2435.00	White, bold
8	EI - 497	G8	81-85	4354.00	White, bold, shiny
9	EH - 1496	G9	80-84	3151.59	White, bold, shiny
10	LM - 10	G10	95-101	2263.00	Yellow, small
11	EI - 561	G11	80-84	4688.00	Yellow, medium size
12	EH -1561	G12	94-100	2424.30	Yellow, small

All the genotypes obtained from AICRP on Maize, MPUAT, Udaipur

1. EI-460 (M) EI-472 (F) EH-1389 (H)
2. EI-628 (M) EI-563 (F) EH-1491 (H)
3. EI-460 (M) EI-497 (F) EH-1496 (H)
4. LM-10 (M) EI-561 (F) EH-1561 (H)

* Common male parent for EH - 1389 and EH - 1496.

The quality of DNA was determined by calculating the ratio between A_{260} and A_{280} which ranges from 1.8-2.0 which is an indicator of moderate good quality of plant DNA and the ratio was almost consistent and irrespective of maize genotypes. The concentration of DNA preparation varied from 1.12 (EI-628) to 2.84 (EH-1389) µg/ml respectively. All the maize hybrids and their parents examined for ISSR genetic marker with 3 primers. Molecular markers data in conjunction to morphological data could be highly useful in precise differentiation and relatedness among the genotypes.

In the present study total 16 ISSR primers (Sigma Aldrich Company) were screened initially with DNA of 3 maize genotypes and finally 3 were selected on the basis of clear, bright and scorable banding pattern. During screening gradient PCR was done in order to optimize the primer annealing temperature (Table 2). The DNA samples were finally diluted to get the final concentration of 25 ng µl $^{-1}$ and subsequently used for Polymerase Chain Reaction (PCR) amplification. The amplification was carried out in a volume of 25 µl containing 200 µm dNTP mix, 1U Taq polymerase, 1X reaction buffer, 0.4 µM primers and 50 ng of template DNA. Amplification was performed in a gradient thermocycler with the following programs: 1 initial denaturation step at 94ºC for 4 min. followed by 40 cycle at 94ºC for 1 min., standardized primer annealing temperature for 2 min. and 72ºC for 2min. and a final cycle of 72ºC for 7min. The amplified products were separated on 2% agarose gel electrophoresis containing 5 µg/ml of EtBr. The gels were photographed under UV light and images transferred to a micro computer for further analysis. A 100 bp DNA ladder was included in the gel as standard molecular weight marker. Each ISSR band was assumed to represent a single locus and data were scored as presence for (1) and for its absence (0).

Electrophoresis pattern of ISSR profile on 2.0 percent agarose gel is illustrated in Plate 1 with three primers. Table 2 illustrates the total number of amplified products. Primer 1 gave 9 scorable bands in the range of ~ 480 to ~ 1200 bp, primer 6 gave 13 scorable bands (~200 bp and ~1300 bp) and primer 8 also gave 13 scorable bands between ~130 bp to ~400 bp. All bands amplified were polymorphic. All the primers showing amplification were repeated twice to confirm the reproducibility and polymorphism. Looking to the Table 2 and Plate 1 eighty four (84) fragments were amplified in all genotypes. 35 scorable amplified fragments were obtained with 3 primers with an average of 11.67 bands per primer. The size of amplification products ranged from 130 to 1300 bp. Different primers showed variation in their ability to detect polymorphism. Primer 1 gave unique band of 480 bp with G3. It gives 9 scorable bands out of which all bands shows polymorphism. Primer 1 gave same banding pattern with G5 and G6. Primer 6 has 13 scorable bands out of which 460 bp is monomorphic in G2, G3, G8 G9 and G12 and absent in G1 (G7), G4, G5, G6, G11. Primer 8 gave 13 scorable bands out of which all are polymorphic. 200 bp is monomorphic to G1 (G7), G2, G5, G6, and absent in G3, G4, G8 G9, G10, G11, G12. A 290 bp band is unique in G4. The primer 6 proved to be best primer in our investigation with total 34 fragments and 13 scorable and polymorphic bands.

The ISSR data were used to obtain a similarity matrix. The similarity coefficient lies upto 0.75. The dendrogram (Fig. 1) clearly indicated that G4 is different from remaining genotypes and these genotypes can be divided in two clusters. The cluster 1 includes G1 (G7), G2, G3, G5, G6, G10 in which G3 and G6 are hybrids. G1 (M) and G2 (F) are parent of G3. G5 is female parent of G6. G1 is related to subclusters, G2-G3 and G5-G6

Table 2. Polymorphism Information of ISSR Primers Analyzed

S. No.	Primer	Sequence 5'-3'	Tm (^{0}C)	Standardized annealing temperature (^{0}C)	Basepair range	Scorable bands	Total No. of polymorphic band (%)	Polymorphism
1.	Primer 1	$(AG)_8C$	46.8	47.8	480-200	9	9	100
2.	Primer 6	$(AG)_8TT$	45.4	48.1	200-300	13	13	100
3.	Primer 8	$(CT)_6A$	26.4	35.5	130-400	13	13	100

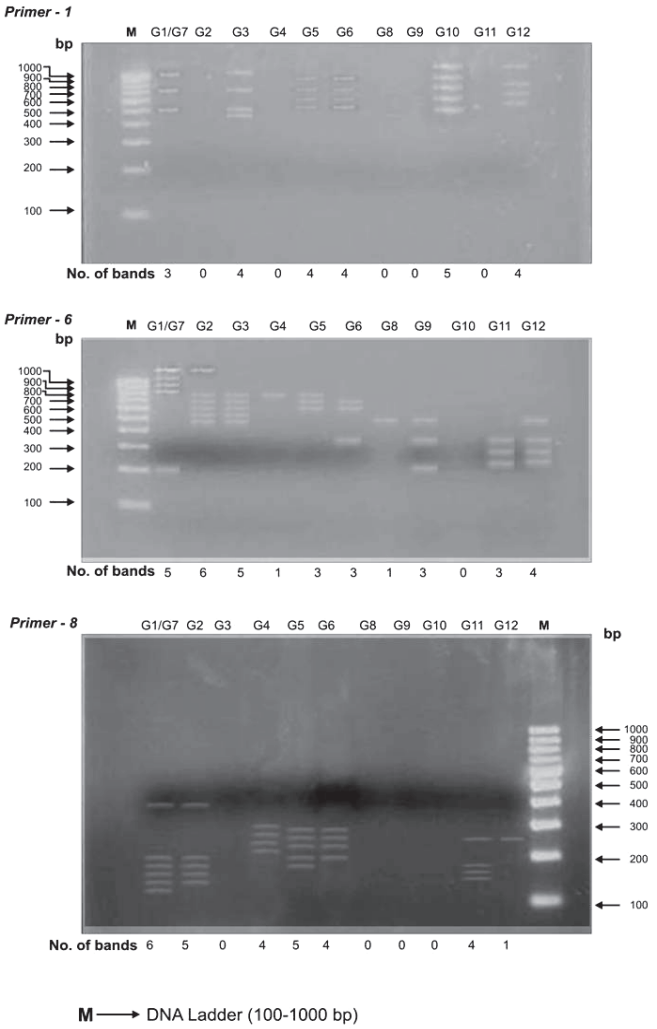

Plate 1. ISSR profile of maize genotypes generated with primers 1, 6 and 8

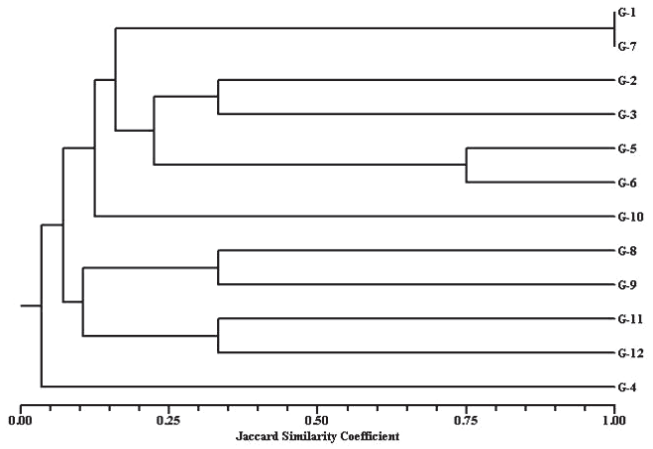

Fig. 1. Dendrogram Generated for Maize genotypes using UPGMA Cluster Analysis Based on Jaccard Similarity Coefficient (ISSR analysis)

with Jaccard's similarity coefficient of 0.15 in cluster 1 subcluster G2 and G3 are related to each other at 0.33 similarity coefficient. This resemblance may be due to the fact that G2 is female parent of hybrid G3. This fact is also applied on G5 and G6. G6 is the hybrid which resemble with G5 (F) with 0.76 similarity coefficient. G10 is related to G1, G2, G3, G5, G6 with 0.13 similarity coefficient.

The cluster 2 includes G8, G9, G11 and G12. It has two sub clusters. Cluster 2 sub cluster 1 includes G8, G9 and sub cluster 2 includes G11 and G12 which are related respectively to each other with 0.33 similarity coefficient. G9 and G12 are hybrid and G8, G11 are female parent of G9 and G12 respectively. G4 and G10 are most different genotypes having lowest germination percentage and yield.

Soluble seed proteins from seeds of maize genotypes, were extracted by using 0.2 M phosphate buffer at pH 7.4 (Seth and Khandelwal 2008) and protein were determined using folin ciocalteau reagent (Lowry *et al*. 1951). The concentration of soluble proteins ranged between 30.0 to 90.0 mg/g of seed flour. Finally the equal amount of lyophilized proteins was subjected to SDS-PAGE (10% and 15%) to generate banding pattern with a protein molecular weight marker [PMW-M (14.3-66.0 kD) from Banglore Genei]. The results revealed a total of 70 and 110 electrophoretic bands on 10% and 15% gel respectively. The size of polypeptides resolved ranged from 11.0 to 59.0 kD on 10% gel and 14.3 to 66.0 kD on 15% gel. For analysis of banding pattern on the gels they were recorded as present or absent. For analysis each band was assigned a value of 1 for presence and 0 for absence.

Protein profile obtained on SDS-PAGE with 10% and 15% gel was used to find genetic relationship among the genotypes. Two bands (23.7 kD and15.8 kD) were monomorphic for all genotypes on 10% gel. On 15% gel also two bands were monomorphic (17.8 kD and 20.1 kD). 24 scorable bands were resolved on 10% gel in

Table 3. Polymorphism Information of protein bands analyzed on SDS - PAGE

S. No.	Gel concentration (%)	Molecular weight range	Scorable bands (a)	Total No. of polymorphic band (b)	Polymorphism (%) b/a x 100
1.	10	11.0-59.0	24	22	91.67
2.	15	14.3-66.0	19	17	89.47
	Total		43	39	90.67

which 22 were polymorphic (91.66%). Similarly 19 scorable bands were resolved on 15% gel in which 17 were polymorphic (89.47%) (Table 3).

The size of polypeptides resolved ranged from 14.3 to 66.0 kD (Plate 2). The polypeptides having molecular weight 27.8 and 20.1 kD appeared in all the genotypes while 37.3 kD appeared only in G9 (EH -1496) and 14.3 kD weight appeared in all except G6 (EH-1491). G9 (EH-1496) produced all bands except 23.7 and 21.1 kD. G8 (EI-497) showed all bands except 37.3 kD. While considering the banding pattern between hybrids and its parents, it showed that EH-1389 which is hybrid of EI-460 (Male Parent) and EI-472 female parent showed same banding pattern at 5 and 7 places with male and female parent respectively. The polypeptide band with

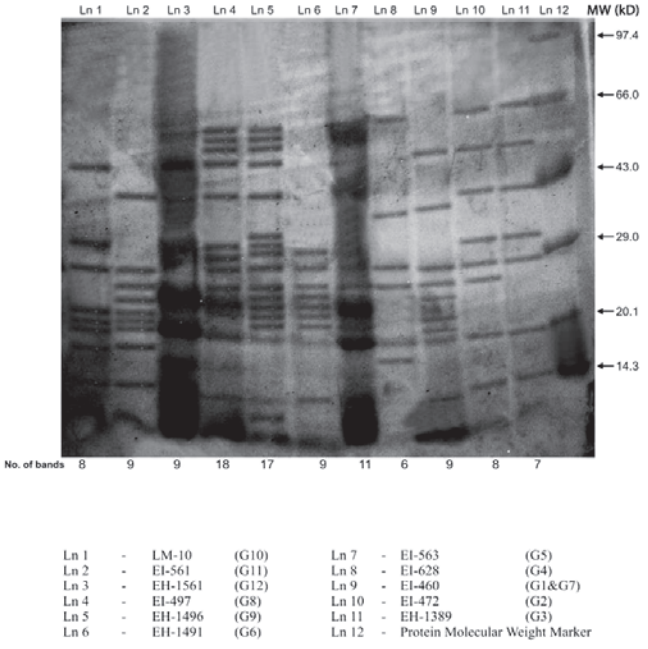

Plate 2. Profile of soluble seed protein on SDS - PAGE (15%)

MW of 26.8 kD is not found in hybrid EH-1389. The polypeptides with 23.7, 22.3 and 21.1 kD MW are found in male parent while absent in hybrid genotype. Morphologically (Table 1) also the hybrids have close similarity with its female parent when crop duration is considered.

G6 (EH-1491) hybrid of G5 [EI-563 (F)] and G4 [EI-628 (M)], showed same banding pattern at 3 places with male parent and at 5 places with female parents. Most of the polypeptides which are found in male parent do not appear in hybrid genotypes. The polypeptides of molecular weight 18.6 kD was found exclusively in hybrid while absent in its parents. G9 (EH-1496) is hybrid of G7 [EI-460 (M)] and G8 [EI-1497 (F)]. The hybrid showed same banding pattern with its female parent at 15 places while it showed similarity with male parent at 7 places. Polypeptides with M.W. 23.7 and 21.1 kD are present in both parents but absent in hybrid. Morphologically also hybrid resembles more with its female parent (height and maturity). G12 (EH-1561) is hybrid of LM - 10 (M) and EI-561 (F). The hybrid showed same banding pattern at 6 and 5 places with female and male respectively. Polypeptides with MW 21.1 kD is present in both parents while 18.6 kD polypeptide is present only in hybrid.

In 10% SDS - polyacrylamide gel 70 fragments were resolved which ranged from 11.0 kD to 59.0 kD out of which 22.1, 33.0, 35.0, 48.0 and 44.0 are unique in G3, 15.0 and 41.7 are unique in G10 and 14.7 and 36.3 are unique in G5. Two polypeptides band of 23.7 and 15.8 kD are monomorphic for all genotypes. Maximum bands are resolved in G5. Band having highest MW of 59.0 kD, is found in G5 and G6. Band having lowest MW of 11.0 kD is present in G3, G5 and G10 like 15% gel, hybrids gave similar banding pattern with the female parent.

Jaccard's similarity coefficient based on SDS-PAGE banding pattern was used for cluster analysis to study genetic relationships. The range of genetic similarity was found to range between 0.21-0.70 (Fig. 2). The dendrogram clearly indicated three main clusters.

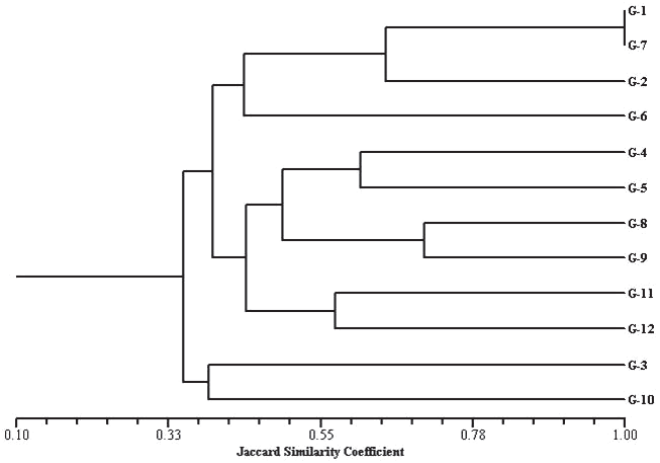

Fig. 2. Dendrogram Generated for Maize genotypes using UPGMA Cluster Analysis Based on Jaccard Similarity Coefficient (Protein profiling)

The clusters 1 included G1/G7, G2 and G6. It has two sub cluster. G1 and G2 are related to each other with 0.64 similarity coefficient. In cluster 2 G4, G5, G8, G9 G11 and G12 are included in which G8 is female parent of G9 hybrid and G11 is female parent of G12 hybrid. G8 related to G9 and G11 related to G12 with 0.70 and 0.57 similarity coefficients respectively (Fig. 2).

At the molecular polymorphism of individual protein amounts (assessed by separation of denatured proteins on SDS-PAGE) were found closely related to genetic distance and hybrid vigour. In addition DNA based markers like RFLP and RAPD, allele specific associated primers, microsatellites, ISSR etc. are being used for assessing genetic diversity among inbred lines. In many studies data from inbreds on DNA markers were used to predict the yield of their hybrids with considerable degree of confidence. ISSR markers are able to differentiate closely related populations (Gonzalez *et al*. 2005). Seed storage protein profiles could be useful markers in the studies of genetic diversity and thereby improving the efficiency of the breeding programme (Shuaib *et al*. 2007). Considerable variation and different genetic responses between genotypes can be analyzed through ISSR and protein electrophoresis. The specific bands could be considered as cultivar specific markers (Abo *et al*. 2010).

Hence study comprising maize hybrids and their parents showed that there was an association between the dendrogram obtained by ISSR markers, protein profiling and morphological characteristics. The hybrids resembled more to their female parent as compared to male parent. Nevertheless, it could be concluded that ISSR and protein profile both are quite efficient in detecting polymorphism and distinguishing cultivars.

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