Indian J. Genet., **61**(4): 304-308 (2001)

Differentiation of elite Indian maize hybrids using simple sequence repeat markers

S. N. C. V. L. Pushpavalli, C. Sudan[1], N. N. Singh[1] and B. M. Prasanna

Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012
[1]Directorate of Maize Research, IARI, New Delhi 110 012

(Received: May 2001; Accepted: September 2001)

Abstract

As an initial attempt towards molecular characterization of maize hybrids released by public sector institutions in India, 10 popular hybrids, including six released and four promising experimental hybrids, were profiled using 20 polymorphic microsatellite or Simple Sequence Repeat (SSR) markers. The utility of SSRs in differentiating the selected hybrids was demonstrated using high-resolution agarose gel electrophoresis. The polymorphic SSR markers identified in this study could clearly distinguish single-cross hybrids developed at Ludhiana, Delhi, Almora and Hyderabad. Cluster analysis based on SSR data delineated the selected hybrids into three distinct groups. While Paras showed considerable genetic similarity with the experimental hybrids developed at Hyderabad, Vivek Hybrid-4 and Vivek Hybrid-5 (from Almora) were found to be genetically distant from other hybrids. The cluster pattern was in close agreement with the pedigree of the parental lines of the single-cross hybrids analyzed in the study.

Key words: *Zea mays* L., hybrids, microsatellite markers

Introduction

Knowledge of the genetic diversity among commercially important maize hybrids can potentially aid hybrid maize breeding strategies by way of planned utilization of promising source germplasm that can enhance genetic diversity. It is now well established that morphological characterization alone does not reliably portray the genetic relationships among the genotypes due to environmental interactions, largely unknown genetic control of these traits, and inadequate sampling of the genome [1]. Biochemical markers such as isozymes, and chromatographic data of zeins, have been used extensively to examine the genetic diversity in commercial maize hybrids abroad [2, 3]. However, limited number of available marker loci and low level of polymorphism are some of the major limitations of the biochemical markers [4, 5]. In view of the possible implementation of plant varietal protection in India in the near future, increasing attention is being paid towards comprehensive characterization of elite Indian maize germplasm, as in other crop species, supplementing the existing morphological descriptors with reliable and repeatable DNA-based marker profiles [6].

SSR markers are PCR-based, codominant, robust, reliable and reproducible, with greater discriminative ability than RFLP or RAPD markers [7, 8]. Several mapped SSR markers are available under public domain in maize [9]. The present study is the first attempt in India towards characterization of a set of important single-cross maize hybrids developed by the public sector institutions, using microsatellite or Simple Sequence Repeat (SSR) markers.

Materials and methods

The experimental material comprised of six released

Table 1. Single-cross maize hybrids analyzed in the present study

S.No.	Hybrid	Parentage	Year of release	Source of seed material
1.	Pusa Early Hybrid Makka-1 (PEHM-1)	CM135 × CM136	1997	IARI, New Delhi
2.	Pusa Early Hybrid Makka-2 (PEHM-2)	CM137 × CM138	1997	-do-
3.	Paras	LM5 × LM6	1995	PAU, Ludhiana
4.	Parkash	CM139 × CM140	1997	-do-
5.	Vivek Hybrid-4	CM212 × CM 141	1999	VPKAS, Almora
6.	Vivek Hybrid-5	CM212 × V25		-do-
7.	BH1073	BIO-1 × BIO-2	Not released	ANGRAU, Hyderabad
8.	BH1117	BIO-3 × BIO-4	-do-	-do-
9.	BH1180	BIO-5 × BIO-6	-do-	-do-
10.	BH1183	BIO-7 × BIO-8	-do-	-do-

and four promising experimental hybrids, representing elite maize hybrids released by different public sector institutions involved in hybrid maize research and development in India (Table 1). All these hybrids, except Paras and Vivek Hybrid-5, were released under the All-India Coordinated Maize Improvement Project (AICMIP) for general cultivation in several states in India. Seed material for the study was obtained from the breeders responsible for the development of the respective hybrids.

DNA extraction from the selected genotypes (leaf samples from at least 20 seedlings) was carried out using the CTAB procedure [10] with minor modifications. A set of 37 SSR primers, selected on the basis of their genomic (bin) locations, were utilized in the present study for analysis of SSR polymorphism among the hybrids. Primers for these SSR markers with different repeat types (di-, tri-, tetra-, penta-, hexa- and compound), were synthesized through Research Genetics Inc., USA, under the Asian Maize Biotechnology Network (AMBIONET), based on primer sequence information available under public domain (Table 2). The PCR amplification cycle consisted of the following steps: initial denaturation at 94°C for 4 min, and subsequent 35 cycles, each with denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 2 min. The final extension step was performed at 72°C for 7 min. The 30µl reaction mix consisted of 30ng of template DNA, 0.25 µM primers (forward + reverse), 0.1mM dNTPs (MBI Fermentas), 0.8U Taq polymerase (Genetaq), 1X PCR buffer, 0.75 mM MgCl2 and deionized water. The amplified products were resolved on a 3.5% SFR (Super Fine Resolution; Amresco) agarose gel that has the capacity to resolve amplified fragments ranging from 75 to 750bp. A 100bp ladder was used for approximate sizing of the amplified products. The protocol followed was mainly based on the method described by Senior and Heun [11], and is also currently being followed by the maize research group at University of Missouri, USA. The gel was run at a constant voltage of 100V for 2h (Bio-Rad Sub-Cell Model 96), in 1X TBE buffer and photographed with a CCD camera (Sony XC-75 CE) attached to a gel doc system (Vilber Laurmat).

Table 2. Maize SSR markers used in the present investigation

S. No.	SSR marker	Repeat type	Bin location
1.	bnlg439	CT	1.03
2.	phi002	AACG	1.08
3.	phi098	AG	2.02
4.	bnlg125	CTGTAG	2.03
5.	phi127	GTCT	2.07
6.	phi099	AC	3.02
7.	phi029	CCCT-CT	3.04
8.	phi079*	CATCTG	4.05
9.	phi093	CTAG	4.08
10.	phi024*	CCT	5.00
11.	bnlg105	AG	5.03
12.	bnlg389	CT	5.09
13.	phi077	AG	6.01
14.	phi070	GAGCT	6.07
15.	phi112	AG	7.01
16.	phi034	CCT	7.02
17.	bnlg572	CT	7.04
18.	phi116	TGAC-GAC	7.06
19.	phi119	AG	8.02
20.	phi125*	AG	8.03
21.	phi033	CTT	9.01
22.	phi042*	CATA	9.04
23.	phi041	AGCC	10.00
24.	phi050	CTTG	10.03
25.	phi062*	GAC	10.04

*Markers that revealed monomorphic pattern in the genotypes analyzed

Scoring of the SSR alleles, sequentially from the largest to the smallest-sized band, was done based on the positions of the bands relative to the ladder. Bands that were either diffused or those that were too difficult to score were considered as missing data (designated as '9' in comparison with '1' for the presence of a band and '0' for the absence of a band in the data matrix). SSR markers showing monomorphic pattern or inconsistent amplification or those with more than 30 per cent missing data were excluded from final analysis. Details regarding the 25 SSRs, including 20 polymorphic and five monomorphic (phi079, phi024, phi125, phi042 and phi062) markers, are provided in Table 2. Amplified products from selected genotypes with distinct or similar alleles were re-run to confirm allele scoring in various hybrids. Jaccard's [12] coefficient (J) was used to calculate the genetic similarities (GS) among pair-wise comparison of genotypes based on SSR data, as follows:

$$J = N_{11}/(N_{11} + N_{10} + N_{01})$$

where N_{11} is the number of bands present in both individuals; N_{01} is the number of bands present only in the individual *i*; N_{01} is the number of bands present only in the individual *j*; and N represents the total number of bands. The similarity matrix was analyzed using NTSYS-pc 2.02 to produce an agglomerative hierarchial classification [13], by employing UPGMA (Unweighted Paired Group Method using Arithmetic Averages) with average linkage [14]. To test the goodness of fit of clustering to a set of data (in this case, the SSR data) 'cophenetic correlation coefficient' or cophenetic value was estimated using the COPH and MXCOMP options in NTSYS-pc program. Canonical discriminant analysis was carried using SPSS

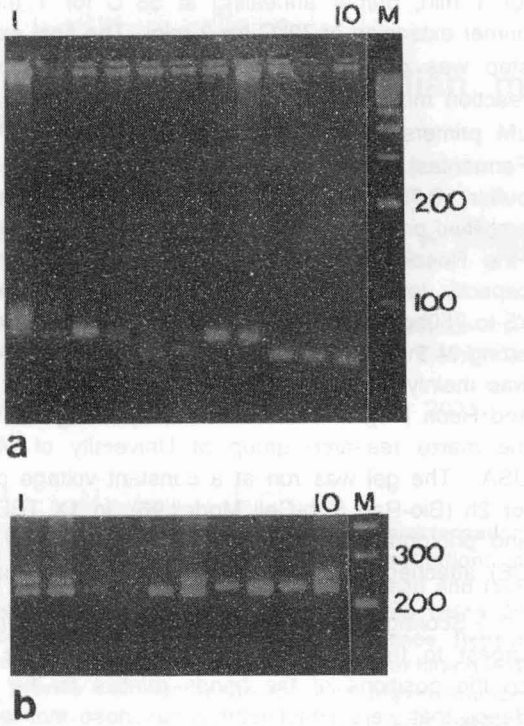

Fig.1. SSR polymorphism in the Indian maize single-cross hybrids revealed using bnig 105 (a) and phi093 (b). The lane information is as follows: PEHM-1 (1); PEHM-2 (2); Paras (3); Parkash (4); Vivek Hybrid-4 (5); Vivek Hybrid-5 (6); BH1073 (7); BH1117 (8); BH1180 (9); and BH1183 (10); M indicates 100bp ladder.

9.0 program for determining the optimal number of clusters.

Results and discussion

Data obtained from the 20 polymorphic SSR loci could clearly distinguish various single-cross hybrids analyzed in the present study (Fig. 1). A total of 38 allelic variants were detected. The SSR markers discriminating different groups of hybrids are presented in Table 3. Vivek Hybrid-4 and Vivek Hybrid-5, developed at VPKAS, Almora, could be clearly distinguished from all the other hybrids by as many as 14 polymorphic SSR markers. Among the primers analyzed, phi033 could distinguish the Vivek hybrids from the PEHM, BH and Ludhiana hybrids. Several markers could also differentiate the four promising experimental hybrids developed at Hyderabad (BH hybrids) from the PEHM hybrids. The markers phi116 and phi127 could discriminate Paras and Parkash from the BH experimental hybrids, while phi034 and phi127 could differentiate the two Ludhiana single-cross hybrids from the PEHM hybrids. SSR markers differentiating the hybrids developed by a specific research institution (PEHMI vs PEHM2; Paras vs Parkash; Vivek Hybrid-4 vs Vivek Hybrid-5) have been identified. A relatively larger number of SSR markers (12) have been found to differentiate the Ludhiana hybrids, Paras and Parkash, highlighting their genetic divergence.

The dendrogram depicting the genetic relationships among the hybrids is presented in Fig. 2. A high

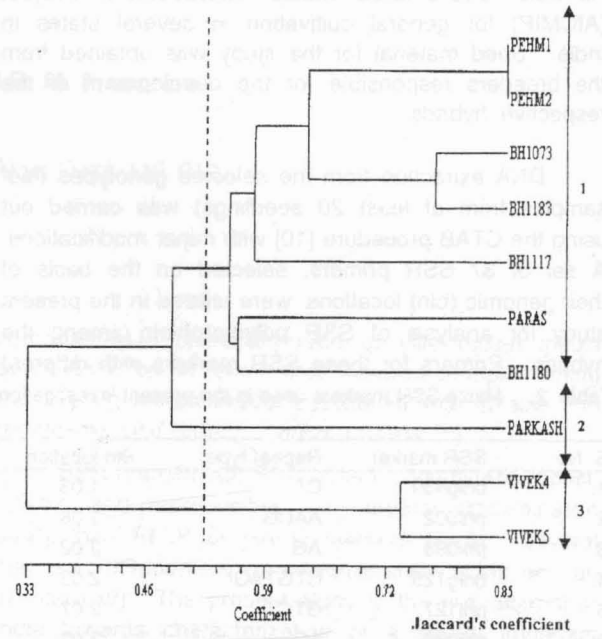

Fig. 2. Dendrogram derived from cluster analysis of SSR data from the single-cross Indian maize hybrids released by public sector institutions.

cophenetic correlation coefficient (r = 0.92) indicated that the dendrogram obtained was a 'good fit' to the similarity matrix generated using the SSR data. On the basis of canonical discriminant analysis the total number of acceptable groups were determined (Fig. 3). Three distinct groups were identified: Group 1 comprising PEHM-1, PEHM-2, BH1073, BH1183, BH1117, Paras, BH1180; Group 2 having Prakash; and Group 3 consisting of Vivek Hybrid-4 and Vivek Hybrid-5. The clustering pattern is largely in consonance with the available information about the source germplasm used in the development of these hybrids. The single-cross hybrids developed at Almora, Vivek Hybrid-4 and Vivek Hybrid-5, appeared to be genetically distinct in comparison with other hybrids. This could be attributed to the breeding history of these lines since the parental lines of the Vivek hybrids were obtained from germplasm specifically adapted to the hill areas such as Pantnagar and Almora in the eastern region of Uttar Pradesh, a major maize-growing state in India. The close genetic similarity between the two Vivek hybrids was also due to sharing of a common female parent, CM212.

Although Paras and Parkash have been developed at the same research center (Ludhiana), the present

Table 3. SSR markers discriminating various single-cross hybrids analyzed in the study

S. No.	Hybrids	Discriminating SSR markers
1.	PEHM (Delhi) vs Paras and Parkash (Ludhiana)	phi034; phi 127; bnlg439
2.	PEHM vs BH (Hyderabad) experimental hybrids	
	- PEHM vs BH1073,BH1117&BH1180	phi034; phi002
	- PEHM vs BH1180	phi041
	- PEHM vs BH1117	phi099
	- PEHM vs BH1180 & BH1183	phi050; phi093
	- PEHM vs BH11117 & BH1180	phi116; bnlg439; bnlg125
3.	PEHM vs Vivek (Almora) hybrids	phi033; phi002; phi099; phi050; phi029; phi041; phi093
4.	Paras & Parkash vs BH hybrids	phi116; phi127
5.	Paras & Parkash vs Vivek hybrids	phi033; phi034; phi050; phi127; bnlgi05; phi070; phi098; phi093; phi077
6.	BH hybrids vs Vivek hybrids	phi033; phi034; bnlg389; phi029; phi070
7.	PEHM-1 vs PEHM-2	phi116; bnlg572; phi077
8.	Paras vs Parkash	phi033; phi034; phi002; phi099; phi050; bnlg389; phi029; phi041; bnlg572; bnlg105; bnlg125; phi077
9.	Vivek Hybrid-4 vs Vivek Hybrid-5	phi127; bnlg572; phi098; phi112

study revealed significant genetic divergence among these hybrids. This could be because of the differences in the source germplasm used in the development of the parental lines. While CM139 and CM140 (parental lines of Parkash) were derived from Indigenous and

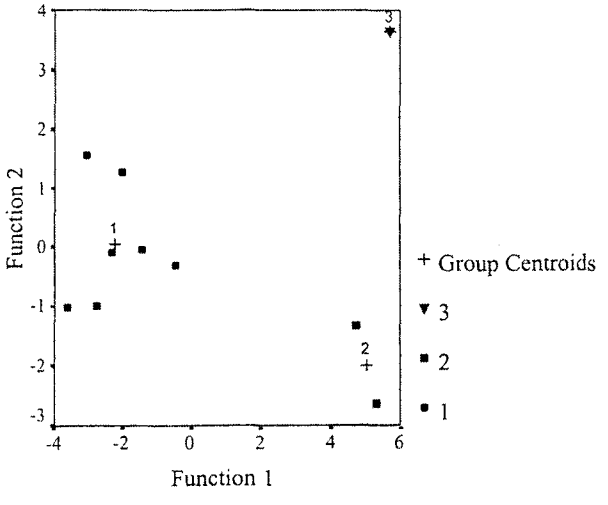

Fig. 3. Canonical discriminant analysis of SSR data to determine optimal number of clusters

Semiexotic heterotic pools respectively, LM5 and LM6 (parental lines of Paras) were developed from Makki Safed-1 and Tuxpeno heterotic pools, respectively [15, 16, 17]. It is interesting to note that Paras showed high genetic similarity with promising BH1180, an experimental hybrid developed recently at Hyderabad. B10-5 is an advanced generation version of LM5 [R. Sai Kumar, personal communication]. SSR analysis of the parental lines of the various single-cross hybrids clearly revealed the close genetic similarity between LM5 (female parent of Paras) and BIO-5 (female parent of BH1180) [18]. SSR data also indicated close genetic similarity between the two PEHM hybrids. CM135 and CM136 (parental lines of PEHM-1) have been derived from A64 and MDR-1 respectively. Population A64 was synthesized from mainly early maturing genotypes, generally exotic germplasm, besides material from CIMMYT. MDR-1 consists of Indian and exotic germplasm. In contrast, CM137 and CM138 were derived from MDR-1 and AD609. AD609 was synthesized using several Indian and exotic early maturing genotypes [V. P. Ahuja, personal communication]. Thus, at least one of the parents of the PEHM hybrids share common source germplasm which could explain the close genetic similarity between the two hybrids.

Several double-cross and double top-cross hybrids have been released by the public sector in India since 1960s, while the first single-cross hybrid, Paras, was released recently in 1995 [15, 19, 20]. Increasing emphasis on hybrid-oriented source germplasm and development of superior inbred lines has resulted in the release of several single-cross hybrids in the recent years. Comprehensive characterization of elite maize hybrids can lead to a better understanding of the pattern of genetic diversity and more effective exploitation of heterotic patterns among germplasm pools. In order to promote independent breeding efforts and to ensure supply of genetically divergent cultivars both in space and time [21, 22], some researchers have recommended implementation of minimum distance criteria [6]. UPOV [23] has particularly indicated the relative advantages of SSR markers over other markers systems such as RFLPs and RAPDs for varietal characterization.

Characterization of hybrids based on DNA-based marker profiles has assumed increasing significance in the context of intellectual property rights [21]. Realizing the large resolution power of DNA profiling, it was generally accepted in UPOV that although the member states at present are not required to use DNA profiling for DUS testing, it could be used in future as complementary information. The present study indicates the utility of microsatellite markers for characterization of the maize hybrids, and provides a possible platform

for generating a more comprehensive SSR database for elite Indian maize germplasm.

Acknowledgment

The first author thanks ICAR for financial assistance in the form of Junior Research Fellowship. The study is carried out with the financial support received by the Indian team under the Asian Maize Biotechnology Network (AMBIONET).

References

1. **Smith J. S. C. and Smith 0. S.** 1989. Comparison of heterosis among hybrids as a measure of hybrid relatedness with that to be expected on the basis of pedigree. Maize Genet. Coop. Newsletter, **63**: 86-87.
2. **Smith J. S. C.** 1984. Genetic variability within US hybrid maize: multivariate analysis of allozyme data. Crop Sci., **24**: 1041-1046.
3. **Smith J. S. C.** 1988. The diversity of US hybrid maize germplasm: isozymic and chromatographic evidence. Crop Sci., **28**: 63-70.
4. **Smith J. S. C., Paszkiewics S., Smith O. S. and Schaffer J.**1987. Electrophoretic, chromatographic and genetic techniques for identifying associations and measuring genetic diversity among corn hybrids. *In*: Proc. 42nd Annu. Corn Sorghum Res. Conf., Chicago, IL. 1987. Am. Seed Trade Assoc. Washington DC, pp 187-203.
5. **Dubreuil P., Dufour P., Krajci E., Causse M., Gallais A. and Charcosset A.** 1996. Organization of RFLP diversity among inbred lines of maize representing the most significant heterotic groups. Crop Sci., **22**: 631-637.
6. **Smith J. S. C., Smith O. S., Bowen S. L., Tenborg R. A. and Wall S. J.** 1991. The description and assessment of distances between inbred lines of maize: 1. A revised scheme for the testing of distinctiveness between inbred lines utilizing DNA RFLPS. Maydica, **36**: 213-226.
7. **Powell W., Machray G. C. and Provan J.** 1996. Polymorphism revealed by simple sequence repeats. Trends Pl. Sci., **1**: 215-222.
8. **Smith J. S. C., Chin E. C. L., Shu H., Smith O. S., Wall S. J., Senior M. L., Mitchell S. E., Kresovich S. and Ziegle J.** 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. Theor. Appl. Genet., **95**: 163-173.
9. **Polacco M.** 1996. Maize genome database. Maize Genet. Coop. Newsletter, **70**: 97.
10. **Saghai-Maroof M. A., Soliman K. M., Jorgensen R. A. and Allard R. W.** 1984. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci., USA, **81**: 8014-8018.
11. **Senior M. L. and Henn M.** 1993. Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome, **36**: 884-889.
12. **Jaccard P.** 1908. Nouvelles researches sur la distribution florale. Bull. Soc. Vaudolse Sci. Nat., **44**: 223-270.
13. **Rohlf F. J.** 1989. NTSYS-pc numerical taxonomy and multi- variate analysis system, version 2.02. Exeter Publications, New York.
14. **Sneath P. H. A. and Sokal R. R.** 1973. Numerical Taxonomy. W. H. Truman & Co., San Francisco.
15. **Dhillon B. S., Malhi N. S., Saxena V. K., Kapoor W. R., Pal S. S., Singh M. and Khehra A. S.** 1995. Paras - first single cross hybrid of maize in India. J. Res. Punjab Agric. Univ., **32**: 238-239.
16. **Khehra A. S. and Dhillon B. S.** 1984. Breeding Maize for Cultivation in Winter. Punjab Agric. Univ., Ludhiana.
17. **Saxena V. K., Malhi N. S., Singh N. N. and Vasal S. K.** 2000. Heterosis in maize: groupings and patterns. *In*: Proc. Seventh Asian Regional Maize Workshop (eds. S. K. Vasal, Gonzalez-Ceniceros, F. and Xingming, F.), PCARRD, Los Banos, Philippines, 124-133.
18. **Sarkar K. R., Singh N. N. and Prasanna B. M.** 1995. Genetic improvement of maize in India. *In*: Sustaining Plant and Animal Productivity - The Challenge of the Decade (ed. D.L. Deb), Associated Publ. Co., New Delhi, 65-82.
19. **Pushpavalli S. N. C. V. L.** 2000. Characterization of Elite Indian Maize Hybrids and their Parental Lines using Molecular Markers. Unpubl. M.Sc. Thesis, P.G. School, Indian Agricultural Research Institute, New Delhi.
20. **Dhillon B. S. and Prasanna B. M.** 2001. Maize. *In*: Breeding Field Crops (ed. V. L. Chopra), Oxford & IBH, New Delhi, 147-185.
21. **Darrah L. L. and Zuber M. S.** 1986. United States farm maize germplasm base and commercial breeding strategies. Crop Sci., **26**: 1109-1113.
22. **Duvick D. N.** 1998. Crop improvement: emerging trends in maize. *In*: Crop Productivity and Sustainability - Shaping the Future. Proc. 2nd Int. Crop. Scl. Congress (eds. V. L. Chopra, R.B. Singh and Anupam Varma), Oxford & IBH Publishing Co., New Delhi, 127-138.
23. **UPOV**. 1997. Working group on biochemical and molecular techniques and DNA-profiling in particular. Fourth Session, Cambridge, UK (March 11-13, 1997). UPOV BMT/4/2/ 1.